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Moderate Heat-Assisted Gene Electrotransfer for Intradermal DNA Vaccination and Protein Replacement Therapy in the Skin

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MODERATE HEAT-ASSISTED GENE ELECTROTRANSFER FOR INTRADERMAL DNA VACCINATION AND PROTEIN REPLACEMENT THERAPY IN THE SKIN

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ABSTRACT

MODERATE HEAT-ASSISTED GENE ELECTROTRANSFER FOR INTRADERMAL DNA VACCINATION AND PROTEIN REPLACEMENT THERAPY IN THE SKIN

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Gene electrotransfer (GET) holds great promise for the delivery of therapeutic agents. The skin serves as an attractive target for GET due to its availability and unique cellular composition. Protein replacement therapy and DNA vaccination are potential applications for intradermal GET. The combination of moderate tissue preheating and GET has been shown to achieve elevated gene expression levels while reducing the necessary applied voltage. In the current work, we utilized a 16-pin multi-electrode array (MEA) and incorporated nine optical fibers, connected to an infrared laser, to pre-heat the tissue to 43°C before application of GET. In a guinea pig model, we found that when the skin was moderately heated, it was possible to achieve the same expression levels as GET at ambient temperature, with a 23% reduction of applied voltage or a 50% reduction of pulse number.

Furthermore, moderate tissue pre-heating allowed for delivery to the deep dermis and muscle, suggesting the potential to reach systemic circulation, a necessary feature for a successful protein replacement therapy. This approach was repeated using a plasmid encoding Human Factor IX, the blood clotting factor defective or absent in patients with Hemophilia B. Elevated Factor IX serum protein levels were detected by ELISA up to 100 days post gene delivery.

The simple engineering and safety of DNA vaccines make them an attractive candidate for alternative vaccine types. Here we present moderate heat-assisted GET for the delivery of a DNA vaccine against Hepatitis B virus (HBV) by way of a plasmid encoding Hepatitis B surface antigen (pHBsAg) via a prime and prime plus boost vaccination protocol. At 18 weeks post vaccination, we observed that a high-voltage low-pulse GET condition with moderate heating (45V 36p +heat) generated antibodies against Hepatitis B surface antigen (HBsAb) at peak measuring 230-fold over an injection only control. Antibody titers remained robust over the 30-week observation period. These data taken together suggest that moderate heat-assisted GET has the potential to achieve systemic delivery with an intradermal approach, an attractive feature for development of both a protein replacement therapy as well as a vaccine delivery platform.

This dissertation is dedicated to my daughter Charlie, for teaching me that the best things in life have their own timing. Thank you for giving me an easy pregnancy, which made all those *in vivo* experiments and long lab hours less strenuous than they might have otherwise been. You've been with me since the beginning of this life chapter, and I half expected your first word to be "electrotransfer". It wasn't, but you can consider this document a testament to your strength as well as my own.

To my husband Bill, thank you for your support emotionally, physically, and financially throughout my graduate career. Thank you for boosting my confidence whenever I started to doubt, for reminding me to put my oxygen mask on first, and for showing up everyday for Charlie and I.

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I give my thanks to all members of the Heller Lab at ODU from 2010-2019. Namely, to Cathryn Mangiamele (Lundberg), thank you for being my right hand during many *in vivo* experiments, ensuring the laser was turned on and off promptly each and every time. I appreciate your dedication and your friendship throughout my time at ODU. To Dr. Anna Bulysheva, thank you for the counsel on matters in and outside of the lab and for teaching me many techniques including immunohistochemistry and the art of cryosectioning.

A special thanks to Dr. Mark Jaroszeski of USF for construction of the novel electrode presented in this work and to James Hornef for technical expertise in laser operation.

NOMENCLATURE

ANOVA	Analysis of variance
DAPI	4',6-Diamidino-2-Phenylindole
DDK	Dbf4-dependent kinase
DNA	Deoxyribonucleic Acid
GET	Gene electrotransfer
H&E	Hematoxylin and Eosin
HBV	Hepatits B virus
HBsAb	Hepatitis B viral surface antigen antibody
HBsAg	Hepatitis B viral surface antigen
IO	Injection only
MEA	Multi-electrode array
OCT	Optimal cutting temperature
pDNA	plasmid DNA
PFA	Paraformaldehyde
pHBsAg	plasmid encoding Hepatitis B viral surface antigen
RTD	Resistance temperature detector

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CHAPTER I

INTRODUCTION

BACKGROUND

Gene electrotransfer

Electrotransfer involves applying an electric field to a cell or tissue to potentiate the delivery of otherwise impermeable aqueous substances. When the electric field is applied, the cell membrane becomes temporarily permeable to a greater extent than when in its native state, a process known as electroporation (1). Extracellular delivery is possible during this quick and transient period. The temporary pores or imperfections in the cell membrane, which enhance its permeability, are resolved once the electric field is removed, allowing normal cellular function to ensue. This approach is referred to as reversible electroporation, where cell viability is maintained. Conversely, in the case of irreversible electroporation, the intent is cellular destruction, which has shown efficacy in cancer therapies and tissue ablation (2-5).

In the method of gene electrotransfer (GET), a plasmid encoding a gene of interest is introduced shortly before application of the electric field through pulse stimulation. The applied pulses cause a direct electrophoretic effect on the injected plasmid DNA, which promotes its migration and cellular uptake. Though the exact mechanism of DNA entry into the cell is not entirely understood, it is clear that the DNA must navigate in some fashion to the nucleus, so it may be transcribed and translated into protein. Recent work in this area of plasmid DNA entry suggests the process happens in several key steps. First, there is permeabilization of the plasma membrane where the DNA is electrophoretically pushed onto and subsequently interacts with the cell membrane. These DNA-membrane interactions or defects remain for up to 10 minutes

after the removal of the pulse. In most cases, the DNA will be internalized by endocytosis and is transported through the cytoplasm. Endosomal escape is the next hurdle for the DNA, after which it must cross the nuclear envelope to be transcribed and translated. The transgene is finally expressed and the newly formed proteins are released into the cytoplasm (1,6). Though this appears to be a treacherous path for the plasmid, current work indicates that cytosolic DNA sensors, such as DNA-dependent activator of interferon regulatory factors/Z-DNA binding protein 1, may play an innate role for DNA trafficking following transfection (7).

Nevertheless, early studies demonstrated successful GET in the rat liver and on brain tumors, indicating enhanced gene delivery efficiency and stability compared to those subjects receiving injection of DNA alone (8,9). Since those experiments, GET has been demonstrated in a myriad of tissue types including smooth muscle, skin, skeletal muscle, and even bone. Likewise, GET has shown efficacy in a number of prophylactic and therapeutic indications including wound healing, cancer treatment, coronary artery disease, metabolic disorders, and vaccination (10-17). There have been 98 studies involving electroporation to make it to clinical trials in the United States, with 41 being currently active. Of those, 36 are for the treatment of various cancers and 14 involve the testing and delivery of DNA-based vaccines with electroporation. (clinicaltrials.gov; Keyword: electroporation, search date: March 1, 2021).

Therapeutic utility of heat

Heat has been used therapeutically since antiquity when Hippocrates recorded the advantages of fever in patients suffering from epilepsy (18). Moreover, in 1927, the Nobel Prize in Medicine and Physiology was awarded to Julius Wagner-Jauregg for his work on the therapeutic value of fever in cases of neurosyphilis (19). Likewise, a host-derived fever is one of

the hallmark immune responses to infection, which has been evolutionarily conserved over millions of years providing a survival benefit despite its associated metabolic or damaging risks. Systemic temperature elevation allows for the activation of effector molecules and facilitates their trafficking throughout the body (20).

In the context of topical therapeutic heat, there are two general categories: deep or superficial (21,22). Deep heat, achieved with ultrasound or microwaves, reaches a penetration depth of 3-5 cm. This modality is preferred for treatment of deep-seated conditions such as Bursitis or Osteoarthritis in the joints (18,22-24). By contrast, superficial heat, such as delivered by electric heating pads, sodium acetate packs, or patches for minor injuries or sprains, is applied to achieve a penetration depth of less than 1 cm (24,25). The results of both therapies are increased blood flow, elasticity of connective tissue, and ultimately pain relief. Though the use of therapeutic heat is quite commonplace, it is critical that both the temperature and duration of exposure be monitored to receive the beneficial effects without the damaging effects (21,25).

There are a number of associated effects of thermal application to consider including sensation, skin discoloration, cellular processes, and even injury (26-29). Based on these data, negative effects from thermal injury begin to occur above 44°C (Table 1). Thermal injury is both temperature and time dependent, where complete transdermal necrosis occurs after just 5 seconds at 63°C while it takes 5000 seconds to induce the same cellular damage at 45°C (25,29).

Table 1. Summary of thermal injury and associated effects *in vivo*. Information included in this table obtained from reference (29).

Sensation	Skin color	Tissue temp (°C)	Cellular process	Tissue/cell injury
Numbness	White	72	Protein Coagulation	Irreversible
	Red & White	64-68	Thermal inactivation	Possibly reversible
Maximum Pain Severe Pain Threshold Pain	Bright Red Light Red	56-60 45-48	of tissue contents	Reversible
Hot Warm	Flushed	44 36-40	Normal Metabolism	None

Skin as a target for GET

The skin is an elaborately organized and highly immunogenic organ. As it is the largest organ of the body, its sheer size and availability make it an excellent target for GET. It is the most visible and therefore exposed organ. The skin serves as the effective barrier between the host and the outside world. Therefore, it functions as the first line of defense against infection and traumas. Skin acts as an insulator, responsible for regulating fluid loss and absorption as well as temperature.

The anatomical structure of human skin is made up of three layers from top to bottom: the epidermis, the dermis, and the hypodermis. The arrangement and thickness of the epidermal and dermal skin layers depend on its location on the body. In general, the thickest

epidermis can be found in thick skin located on the palms and the foot sole measuring approximately 2 mm (30,31). By contrast, skin found on the arm for example has an approximate epidermal thickness of 0.8-1 mm. The epidermis is made primarily of keratinocytes, but also includes melanocytes, Merkel cells, and antigen-presenting dendritic cells called Langerhans. The epidermis is made up of five bands: the stratum corneum, the stratum lucidum, the stratum granulosum, the stratum spinosum, and the stratum basale. Cells are formed at the basal membrane and migrate upwards as they mature. The turnover rate for the entire epidermal skin layer is approximately 27 days, with the outermost layer of epithelial cells sloughing off after two weeks (30,31).

The dermis is located beneath the epidermis and provides the circulatory support and nutrition for the epidermis through communication at the epidermal-dermal junction. The dermis includes cells such as fibroblasts, monocytes, Langerhans cells, lymphocytes, and eosinophils, but is mainly comprised of connective tissue (31). Blood and lymph vessels can be found in the connective tissue matrix of the dermis. These vessels offer support to the epidermis above and to the hypodermis or subcutaneous tissue below.

The skin is a feasible target for GET. It is accessible, easily monitored, and its highly immunogenic properties enhance its utility for the delivery of therapeutic agents. Preclinical studies have used mice, rats, guinea pigs, pigs, and non-human primates as models for skin research (32-34). The guinea pig serves as a good model for human skin because it has anatomical similarities. Cellular composition in the dermis including Langerhans cells, dermal fibroblasts, and macrophages make it a good model. Also, guinea pigs have a comparative thickness to human skin.

Two categories of electrodes have been developed for use in skin: penetrating and non-penetrating. Penetrating electrodes consist of needles arranged in various configurations that are used to pierce the stratum corneum for pulse delivery. The applied voltage from these penetrating electrodes is dependent on design, but ranges anywhere from 50-1750V/cm. Penetrating electrodes have been evaluated as a platform for DNA vaccination demonstrating adaptive immunity compared to direct injection of plasmid DNA (35).

Non-Penetrating electrodes provide a non-invasive alternative, where the electrode does not have to be inserted in the skin for pulse application. Caliper and tweezer-type electrodes were the early configurations of non-penetrating electrodes, which applied pulses in a single direction. By contrast, four plates oriented in a square pattern could deliver pulses in multiple directions (36). Though effective for gene electrotransfer, the large gap between electrodes required high-voltage or high-pulse number to achieve elevated levels of gene expression. In some cases, the high-voltage or high-pulse number applied induced superficial skin damage in preclinical testing and presumably pain. Furthermore, the geometry of some of the configurations provided limited coverage of the target in certain cases.

As a solution to this problem, the multi-electrode array (MEA) was designed providing a short gap between electrodes, thus effectively reducing the required voltage. This created a non-penetrating minimally invasive approach to cutaneous gene delivery. The MEA has shown efficacy in a number of preclinical studies including DNA vaccination against *B. anthracis* and Hepatitis B virus, wound healing, and Factor IX protein delivery (37-40).

DNA vaccines

Current vaccines on the pharmaceutical market are comprised of killed whole pathogen,

pathogen subunits, or live-attenuated viruses. Killed whole pathogen or subunit vaccines confer their protection primarily through a CD4+ T-cell response and innate immune mechanisms. This type of response generally does not provide life-long immunity. Several immunization boosters are thus needed to maintain what is presumed to be adequate protection. Live-attenuated or weakened viral vaccines are able to induce both the innate and adaptive arms of the immune response. However, because the virus has been attenuated it inherently is less immunogenic. Therefore, inducing a more prolonged immunity and requiring less frequent booster immunizations would be advantageous. Also, the aim to include protection from several pathogens within one vaccine is appealing, but is difficult to obtain with a live-attenuated platform. Safety concerns such as reversion to a live state or allergies, which exclude some population cohorts, remain with current vaccine types. These limitations and others motivate the development of alternative vaccine platforms.

DNA vaccines provide an attractive alternative. They are non-live and non-replicating and specifically encode the antigen or antigens of interest. In the early 1990s, DNA vaccinations were reported to induce antibody responses to viral or bacterial antigens when delivered to skin or muscle (41-43). In principle, DNA vaccines were able to generate robust immune responses without the need of a replicating pathogen to purify them. Success in preclinical studies helped facilitate the translation of DNA vaccines to clinical trials. The first trials evaluated the efficacy of a DNA vaccine against HIV-1 (44). Criticism of DNA vaccines questioned the risk of genome integration. There is little evidence of integration observed from DNA vaccines, and even appears to be lower in risk than that of naturally occurring genetic mutations (45,46). The list of pathogens successfully targeted by DNA vaccination in clinical

trials and preclinical studies have included influenza, Human Papilomavirus, Hepatitis B, Malaria, Zika virus, SARS-CoV, Ebola, and West Nile Virus (47-52). There is even a current ongoing clinical trial evaluating the use of a DNA vaccine against SARS-CoV-2, the causative agent of the COVID-19 pandemic. Though safe and well tolerated, results of the completed studies suggested that injection of plasmid DNA alone was not very immunogenic (47-52).

GET can serve as an adjuvant to enhance the immunogenicity of DNA vaccines. Early data suggested that humoral responses and cell-mediated immune responses to DNA vaccines against HBV and HIV gag protein respectively were induced via GET-mediated delivery (53,54). Since, GET has shown efficacy for stimulating an immune response, additional viral pathogens such as HIV, HBV, West Nile Virus, and SARS-CoV-2, Human Papillomavirus, and Influenza have been tested using a GET-delivered DNA vaccine (49,55-62). Taken together, these results demonstrate the potential of GET to enhance the delivery DNA vaccines as well as their immunogenicity compared to direct injection.

Primarily, the use of invasive penetrating electrodes has been employed for GET-mediated delivery of DNA vaccines. Muscle is the primary site for these applications, as it is highly vascularized and able to express DNA for a long time compared to other tissues (15,63,64). Delivery to the muscle can be painful, so the skin has been used as an alternative site for DNA vaccination using non-penetrating electrodes (38,40,65,66).

Protein replacement therapy

Protein replacement therapy involves the supplementation of or replacement of a protein which is either deficient or absent due to a genetic abnormality. Protein replacement has been studied in a wide variety of conditions including blood disorders, metabolic disorders,

and lysosomal storage disorders (67). Gene therapy has shown promise in the control and treatment of these disorders.

The most promising data has been shown in the treatment of the blood clotting disorder Hemophilia (68). Hemophiliacs are missing or deficient in clotting proteins Factor VIII or Factor IX for Hemophilia A or B respectively. Before the 1960s life expectancy of someone with severe Hemophilia was less than 20 years old (69). It was at this time that the infusion plasma products were used to stabilize the disease, however the overall concentration of clotting factors actually in human-derived plasma was and is still very low. Later that decade, it was found that clotting factors could be precipitated and pooled from plasma and directly infused. During the HIV/AIDS epidemic in the 1980s it was found that nearly half of Hemophiliacs had contracted AIDS, presumably from plasma-derived products. The identification and sequencing of both of these proteins in the 1990s perpetuated the use of recombinant Factor VIII or Factor IX injections and fusions, providing an alternative that was not plasma derived (69,70). The current standard of care is recombinant protein, where prophylactic approach is taken requiring 2-3 infusions each week. Though effective and life saving, high doses are required to achieve systemic levels, as are the aforementioned frequent administrations.

Conversely, utilizing gene therapy for this indication can induce sustained protein expression levels, increasing the time between repeat administrations (71). Viral vectors, such as recombinant adeno-associated virus (rAAV), have shown some efficacy for the delivery of Factor VIII and IX proteins (70). Though able to induce high levels of transgene expression, the use of viral vectors may not be suitable for all patients. A humoral immune response via neutralizing AAV antibodies is a concern, as approximately 40% of the population has pre-

existing antibodies against AAV (72). This effectively reduces the protein expression potential in those patients and eliminates them as potential therapy recipients. Also, a cell-mediated immune response has been shown in response to the AAV capsid, evidenced by AAV capsid-specific CD8+Tcells (73,74). This not only was shown to cause reduced expression potential, but also clearance of transduced hepatocytes in liver-directed rAAV delivery (68,75).

Using the skin as an alternative delivery organ for protein replacement therapy is advantageous. In the case of Hemophilia, where severe bleeding is a real concern, a minimally invasive approach is crucial. A non-penetrating electrode is thus an attractive platform. Pre-clinical studies in hairless guinea pig displayed elevated Factor IX protein levels using the MEA for gene electrotransfer compared to injection of plasmid DNA alone (39). However, protein levels were much lower than therapeutic levels suggesting this approach would be more suited for the delivery of low dose protein products such as growth hormone.

RATIONALE AND HYPOTHESIS

The effect of temperature on cells and biological tissues has been well studied, where an increase in temperature yields an increase in cell permeability (76,77). This membrane dilation can facilitate the transfer of otherwise impermeable deliverables, such as plasmid DNA, hormone, or chemotherapeutic drugs to cells or tissues (37,78,79). When the application of moderate heating and electric field is combined, these effects are more pronounced and delivery is more efficient (Figure 1). Another consequence of heating is irreversible electroporation, where the heat sensitizes cancer cells to the applied electric field, thus making a more effective treatment (3,4).

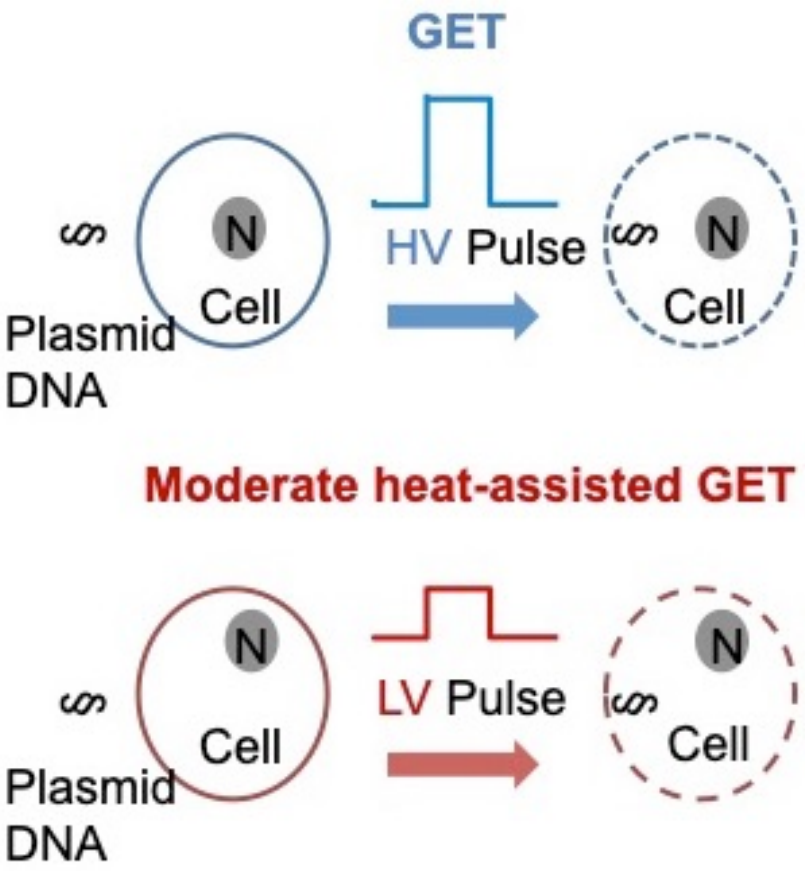

Figure 1. Schematic theoretical representation of moderate heat-assisted GET. GET is considered a nonthermal process requiring a high voltage pulse to deliver plasmid DNA through transient perturbations in the cell membrane. The use of moderate heat reduces the required voltage for GET. The membrane becomes more fluid under moderate heat allowing for delivery at the lower applied voltage.

In the current work, we present a platform for reversible electroporation, where cell viability is maintained. We present utility of the minimally invasive non-penetrating 16-pin MEA with the incorporation of an infrared laser for moderate heating of the target tissue. We heat

the skin to a moderate temperature of 43°C for a total of 30 seconds and maintain an elevated temperature for an additional 20 seconds after the exogenous heat source is removed. At this temperature, 41-43°C, the skin is temporarily warm and normal metabolism still proceeds. An infrared laser was chosen as the heating source in this case due to its speed and precision at which the target temperature could be reached.

In our previous work, a four pin MEA was utilized with a centrally located infrared laser shining out of a single optical fiber for moderate heating. This worked well as a proof of principle, but the gap between the electrodes and laser beam path, suggested moderate heating coverage of the target tissue could be improved (78). In addition, an applied voltage of 50V was required for thermally-assisted GET. By utilizing a 16-pin MEA we are able to reduce the electrode gap due to its geometry and thereby reduce the necessary applied voltage for gene delivery. Also, the 2 mm gap between electrodes left enough spacing to include nine optical fibers. In theory, this allows for greater coverage of the target tissue than when using a single fiber. Combined, the use of moderate heating and a shorter electrode gap suggested the potential to reduce electrotransfer conditions even further all while maintaining or enhancing gene expression.

The current design is novel compared to our previous work, with the infrared laser split equally among nine interspaced optical fibers. We hypothesize that this configuration will yield more uniform heating compared to our previous design and allow for reductions in either necessary applied voltage and/or pulse number. We tested this device for the delivery of reporter genes to assess both the kinetics and distribution of gene expression following moderate heat-assisted GET. We hypothesize that the addition of moderate heating

incorporated into the 16-pin MEA would yield higher expression levels compared to its unheated counterpart and that moderate heating would facilitate applied voltage reduction.

A minimally invasive platform is advantageous for non-life-threatening applications such as vaccination. Furthermore, an approach that generates a robust systemic response will require less frequent booster immunizations. To determine the potential of utilizing moderate heat-assisted GET for DNA vaccination, a DNA vaccination protocol against HBV was carried out. We have previously shown efficacy in utilizing the MEA for GET of a plasmid encoding Hepatitis B surface antigen to achieve detectable neutralizing anti-HBs. We sought to improve upon that ambient platform with our moderately heated platform. High-voltage low-pulse number and low-voltage high-pulse number conditions were mediated with the addition of moderate heating. We hypothesize the addition of moderate heating will result in elevated antibodies to HBsAg detected in serum compared to its unheated counterpart.

Lastly, we sought to evaluate moderate heat-assisted GET for the cutaneous delivery of human Factor IX in an *in vivo* guinea pig model. Like vaccination, a minimally invasive platform is ideal for Factor IX replacement therapies as frequent administrations are typically required. The aim would be to create a less painful and less frequent treatment plan. We previously demonstrated delivery of Factor IX in hairless guinea pigs using the MEA. Here we expand upon this work with the addition of heat, as those early studies indicated that the amount of Factor IX to reach circulation was quite low. We hypothesize that the addition of moderate heating will facilitate the delivery of Factor IX via intradermal GET to reach systemic levels rather than just local.

CHAPTER II

MODERATE HEAT-ASSISTED GET DEVICE

ELECTRODE ASSEMBLY AND MODERATE HEATING PARAMETERS

GET device

Moderate heating and GET delivery were incased in a single novel device for ease of use in a streamlined approach. Providing one device for this application facilitates its translation for future use in a clinical setting. The design of the Multi-electrode Array (MEA) GET delivery device has been previously described (36,80,81). The electrode included 16 spring-loaded gold-plated round-tipped pins arranged in a 4x4 square pattern 2-mm apart from one another.

GET delivery parameters

Each pair of conductive electrodes was programmed to administer either a train of two or four pulses. In the current work, these pulse trains were administered as four pulses 18 times for a total of 72 pulses or two pulses administered 18 times for a total of 36 pulses depending on desired GET conditions. The applied voltages used for this study were either 35 or 45V between two conductive pins, where each pulse had a duration of 150 ms with a 150 ms delay between pulses. Electrotransfer pulsing was performed using the UltraVolt Model: Rack-2-500-00230 (UltraVolt, Inc. Ronkonkoma, NY, USA).

Infrared laser system for moderate heating

An infrared radiation emitter was integrated into the design of the MEA GET applicator. Though the moderate heating and GET delivery was incased in a single handheld device, moderate heating was always applied before GET in these experiments, as a way to pre-heat the target tissue. Moderate heating was applied to maintain a surface temperature of the

treatment site tissue at 43°C. The heating system consisted of a 980 nm wavelength infrared laser (Lasermate Group Inc, Walnut, California), an optical fiber that delivers the infrared radiation laser light to the skin, and an emission splitter. The infrared laser emitter consisted of an optical fiber whose beam was split equally among nine individual optical fibers that utilized four separate splitter boxes (Figure 2). The primary optical fiber was connected directly to a three-way splitter box, which had three fibers each connected to another three-way splitter box. Each connection to the splitter boxes had a shutter than was open or closed manually to control the laser beam.

The nine optical fibers are positioned equidistant and centrally between 16 0.5mm gold-plated round tipped pins of the MEA each spaced 2mm apart, creating a combined 6 by 6 mm moderate heating electrode array (Figure 3). As previously shown, the small size and dielectric material of the optical fiber can be integrated into a GET delivery system without causing electrical interference [69].

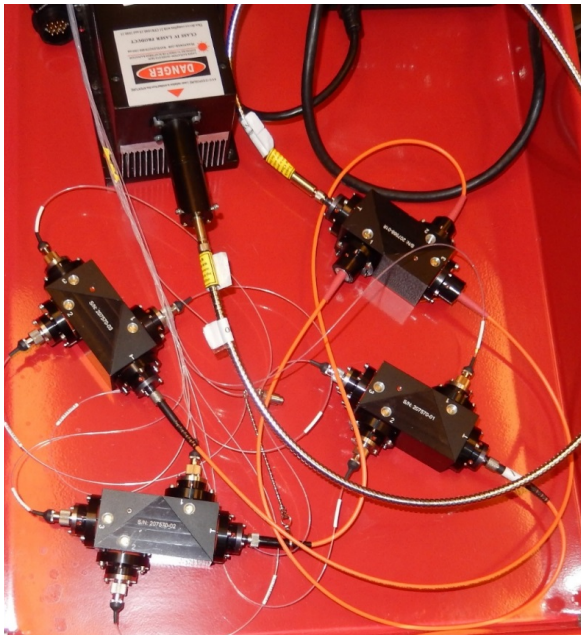

Figure 2. Infrared laser heating system for moderate heat-assisted GET. The main optical fiber for moderate heating connects to a primary three-way splitter box. Three secondary splitter boxes are then connected each by via three separate optical fibers to the primary three-way splitter box (orange wires). Each of the three secondary splitter boxes directs the laser beam through three optical fibers all connected to the main optical fiber. The controlling the opening and shutting of nine individual fibers is performed manually by nine individual shutters.

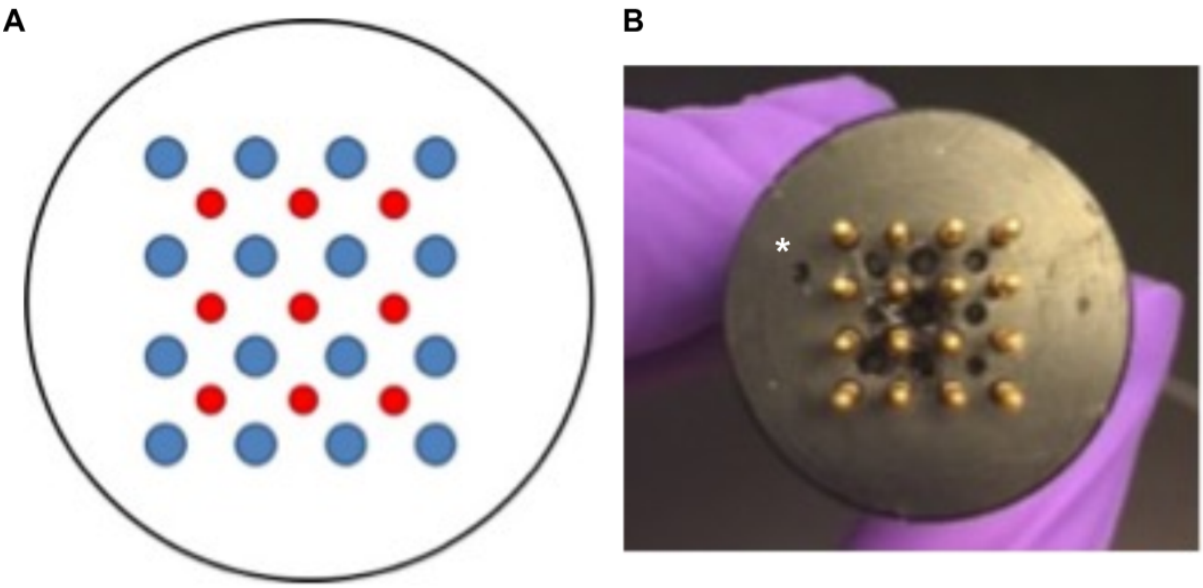

Figure 3. Moderate heat-assisted GET device. (A) Moderate heating is performed by an infrared laser split through nine equally spaced optical fibers (red circles). GET is applied from spring-loaded pins placed in contact with skin target (blue circles). (B) In reality, the 16-pin MEA with evenly interspersed nine optical fibers utilized for moderate heat-assisted all encased within a non-conductive Teflon cap. Blank pin at left of image (*) indicates a place marker for maintaining uniformity in placement of the electrode.

MODERATE HEATING PARAMETERS EX VIVO

Ex vivo pig skin model

Porcine skin was acquired post-mortem from an existing IACUC approved protocol with proper permissions. Skin was removed from the flank and void of excess hair. The skin sections were then embedded in Optimal Cutting Temperature compound and frozen at −80 °C. To obtain serial sections, individual pieces were mounted en face and cryosectioned serially

starting from the outermost epidermal side. A 200µm serial depth pattern was performed on the cryostat. This allowed us to profile heating at depths of 200µm, 400µm, and 600µm.

All laser heating calibrations were performed by placing the electrode casing with integrated laser in a micromanipulator and aligning the laser with a resistance temperature detector (RTD) thermocouple (Figure 4). Once the laser was aligned, the location was fixed. This set-up reduced the error that could be attributed to any unsteadiness in the holding position. The laser power supply was set to 3 amperes for these experiments yielding an ideal fast rise-time. Serial pig skin sections were carefully stacked with outermost epidermal layer on top. A RTD thermocouple was sandwiched in-between each layer in subsequent experiments in order to record the temperature at specific depths before, during, and after laser heating.

Temperature distribution at sequential depths of 200µm, 400µm, and 600µm were recorded. Temperature measurements were taken at a rate of 5 reads per second, with the resulting temperature value being an average of these 5 readings. Baseline temperature was measured prior to turning on the laser for heating. For the outermost skin depth, 200µm, the laser was turned off once the temperature at the RTD thermocouple reached 43°C and the time for temperature decay was measured. For subsequent depths, the thermocouple was moved to the next deepest layer and the laser was turned off at the same time that the first depth reached 43°C. Likewise, the temperature decay was followed. At each sequential depth, the entire stack was shifted slightly, ensuring the same region was not directly heated each time.

Baseline temperature of the pig skin sections were recorded as 21.6 ± 0.3°C at 200µm, 21.9 ± 0.6°C at 400µm, and 21.3 ± 0.5°C at 600µm. At a 200µm depth, the laser was able to heat pig skin to 43°C in 6 seconds after turning on the laser and setting the power to 3 amperes. At

this same time point the temperatures at 400µm and 600µm were 36.0 ± 4.4°C and 34.7 ± 2.6°C respectively. The temperature gradually declined and returned to baseline after 35 seconds (Figure 5).

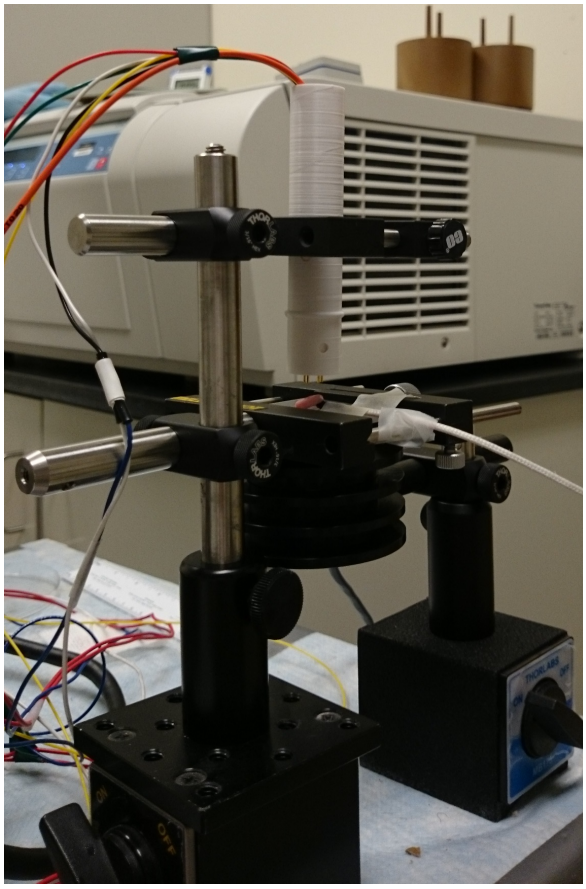

Figure 4. Laser heating calibration set-up. A micromanipulator was used to obtain steady measurements of laser heating in an ex vivo model. Serial pig skin sections were stacked on top of an RTD thermocouple that was used to obtain temperature measurements. This set-up was repeated for all tested samples.

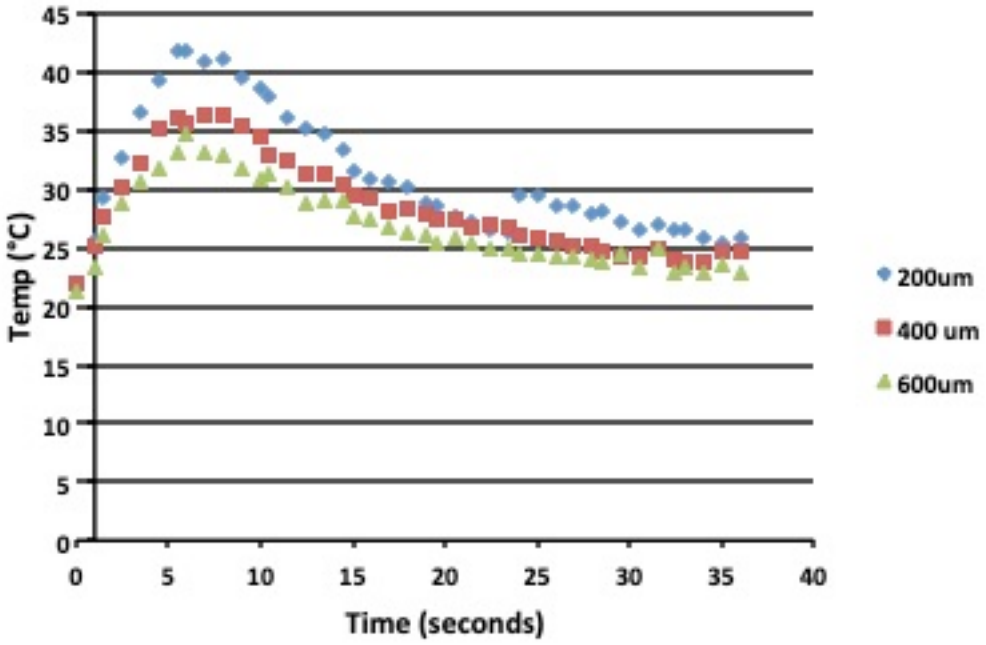

Figure 5. Depth of laser heating in an ex vivo porcine skin model. Frozen pig skin was sectioned serially at 200µm depths on an upright cryostat. Serial pig skin sections were stacked with outermost epidermal face on top. A thermocouple was placed at subsequent 200µm depths in-between each graft to obtain temporal temperature measurements following laser heat application: 200µm, 400µm, and 600µm. All experiments were performed with the laser attached to a micromanipulator to prevent excess movement and ensure alignment for the duration of the recorded time. Temperature measurements were taken at 5-second intervals for a total of 35 seconds. A target temperature of 43°C was reached before turning off the laser, after which temperature decay was recorded. Data represent average temperature (°C), n=5 individual stacks.

Recellularized dermis

Recellularization of human dermal constructs was performed using an established protocol (82). Briefly, DermACELL® (LifeNet Health, Virginia Beach, VA) grafts were washed in DPBS and cut into 12mm disks using a biopsy punch, then washed twice with complete DMEM media. Human primary dermal fibroblasts (ATCC, Manassas, VA) were maintained in culture with DMEM, 10% fetal bovine serum, 1% Penicillin/Streptomycin at 37°C and 5% CO_2. Culture medium was replaced every 2-3 days. HaCaT cells were maintained in culture using both the same media formulations and growth conditions as the fibroblasts.

Fibroblasts were seeded at a density of $1x10^6$ cells/disk on the reticular face of the dermis. After fibroblast attachment, the constructs were transferred to fresh wells. Fibroblasts were maintained on the dermis for one week, allowing for cell infiltration throughout the matrix. On the papillary face, HaCaT cells were seeded at a density of $1.5x10^5$ cells/disk. HaCaT cells attached overnight, and the grafts were moved to the upper chamber of Transwell Permeable Supports (Corning Incorporated, Life Sciences, Tewksbury, MA). The bottom well was filled with fresh media, while the top well was left void of any media, effectively lifting the grafts to an air-liquid interface (Figure 6). This action allowed for epithelial cell stratification. Recellularized dermis was cultured for 3 weeks with daily media changes before applying moderate heating.

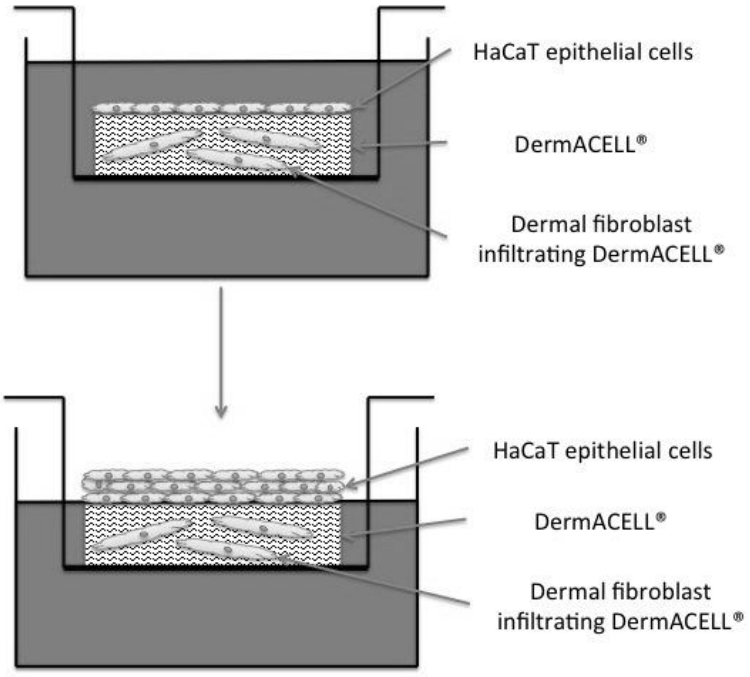

Figure 6. Diagram of air-liquid interface culture of recellularized dermis. Dermal constructs were cultured at an air-liquid interface to encourage stratification of epithelial cells. Complete DMEM media in the bottom well was replaced daily and excess moisture removed from the top of the Transwell. Figure reproduced with permissions from reference (82).

Moderate heat calibration in a 3D human skin model

Before advancing to an *in vivo* model, the novel moderate heating set-up was assessed in recellularized human dermal constructs. The recellularized human skin grafts were 3.5 weeks old in culture when used for this experiment. The grafts were carefully removed from their transwells using forceps, so as not to disrupt the newly formed epithelium. The graft was

dabbed dry using sterile gauze to remove excess fluid from both sides of the graft. The graft was injected intradermally with 50µL of 0.9% physiological saline in the center of the graft. The graft was then placed, with the reticular side facing up, directly on top of the RTD thermocouple, which had been pre-aligned with the laser fiber at a distance of 1.5cm from the graft. Temperature measurements were also taken every 5 seconds. Baseline temperature was measured prior to turning on the laser for heating. The laser was turned off once the temperature reached 43°C, and the time for temperature decay was measured.

After heating, the recellularized human skin grafts were fixed in 4% PFA. For storing, the samples were placed in a gradient of Optimal Cutting Temperature (OCT) compound overnight on a rotator ensuring proper fixation. The grafts were then frozen in OCT and stored at -80°C until sectioned. Grafts were then sectioned on a cryostat to determine the thickness of each graft. A hematoxylin and eosin (H&E) staining protocol was performed to visualize the graft sections (Figure 7).

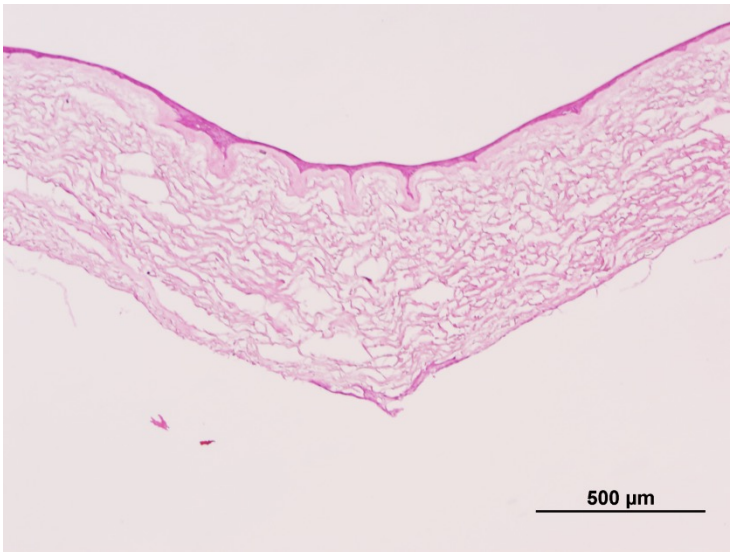

Figure 7. H&E staining of recellularized dermal grafts**.** An H&E staining was performed on recellularized dermal grafts following infrared laser heating. Samples were fixed in 4% PFA, placed in OCT, and frozen until sectioning. Grafts were sectioned on an upright cryostat. All micrographs were taken on a Leica systems inverted microscope. Scale bar = 500µm, magnification = 40x.

Using the measuring tool in IMAGEJ, a scale was set for each image using the existing scale bar. Three separate regions of the stained tissue were measured from epidermal to dermal face and averaged to obtain average graft thickness (Table 2).

Table 2. Recellularized dermal graft thickness. Recellularized dermal constructs cultured for three weeks were fixed and stained using an H&E protocol. Micrographs were taken on a Leica systems inverted microscope and imported into IMAGE J for measurements. Three separate regions on the graft were identified, measured, and averaged to determine graft thickness (μm). The standard deviation (stdev) between the three measurements were recorded.

	Recellularized dermis thickness (μm)				
Sample ID	Measure 1	Measure 2	Measure 3	Average	Stdev
1	416.56	417.83	447.13	427.17	17.29
2	355.85	363.64	388.31	369.27	16.95
3	348.43	357.23	373.59	359.75	12.77
4	570.97	706.45	619.36	632.26	68.66
5	575.96	699.43	560.14	611.84	76.26
6	525.79	518.25	505.66	516.57	10.17

Baseline temperature of the recellularized dermal skin grafts was recorded as 18.3 ± 0.1°C, with the skin graft placed on top of the RTD thermocouple (Figure 8). It took 20 seconds for the skin graft to be heated to 42.0 ± 1.2°C. The depth of this temperature reading varied depending on the thickness of the graft.

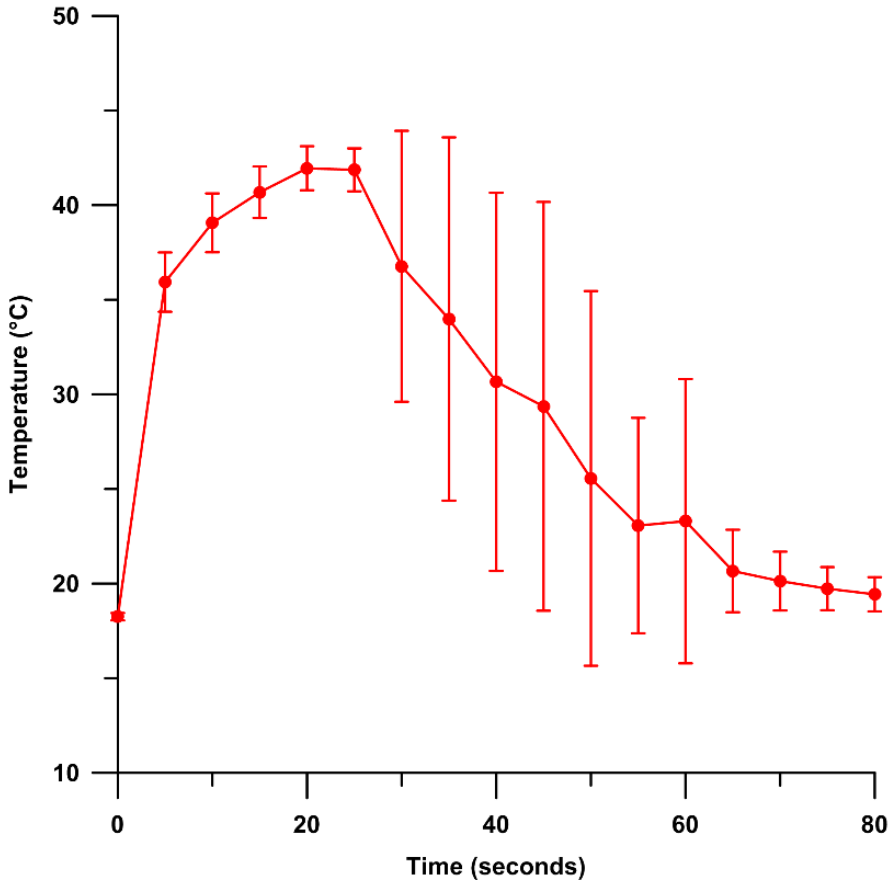

Figure 8. Temporal heating of recellularized dermal grafts. An infrared laser was used to heat the 3D human skin model to 43°C. Temperature measurements were taken via a thermocouple placed underneath the graft aligned with the output laser beam. Temperature measurements were recorded in 5-second intervals. The laser heat source was turned off once the underside of the graft reached 43°C. After which, the temperature decay was monitored until returning to baseline. The data represent the mean temperature (°C) ± standard error of the mean (SEM), n=6 individual grafts.

MODERATE HEATING PARAMETERS IN VIVO

Animals

The *in vivo* animal model used for this study was the guinea pig, as guinea pig skin shares similarities in thickness and composition with human skin. Animals used for this study were 8-10-week-old Female Hartley guinea pigs weighing approximately 300-350g at the time of treatment. The animals were housed at the Old Dominion University animal facility and procedures approved by the Old Dominion Institutional Animal Care and Use Committee approval #17-022. All animal subjects were quarantined for a minimum of 7 days following arrival and prior to the conduction of any procedures.

Moderate heat application and duration

All animals were anesthetized during moderate heating. Anesthesia was performed by placing the animals into an induction chamber infused with 3-4% isofluorane and 96-97% oxygen gas. After animals were sufficiently anesthetized, they were fitted with a standard rodent mask supplied with 3% isofluorane and oxygen to maintain a surgical plane of anesthesia for the duration of the procedure. Animals were placed on a heating pad to ensure thermoregulation during the entire procedure. Animals were then shaven and washed with mild soap (Dial) and water in an outward circular motion to remove any loose hair or an overabundance of oil.

Prior to utilizing the addition of moderate heat in combination with the administration of GET, the time needed to raise the temperature of the dermis using the infrared laser in this novel configuration was determined. Temperature measurements were accomplished by inserting an 18G needle intradermally into either the pre-shaven left or right flank then

carefully replacing the needle with a K-type thermocouple temperature probe (Omega, Stamford, CT, USA), which was guided through the needle shaft and out the bevel. Temperature was recorded via a digital output.

Baseline temperature in an anesthetized guinea pig kept under thermoregulation via a heating pad was determined to be 35°C. For moderate heat application, the electrode was placed with the spring-loaded GET electrode pins in contact perpendicularly with the skin, leaving a fixed distance of 1 cm between the optical fibers and the skin target. Gentle pressure was applied to ensure uniform contact. With the thermocouple in place, temperature measurements were then obtained during the application of moderate heat. Safety precautions such as eyewear and barriers were in place during all laser operations.

We assessed the heating capabilities of three, six, and nine operating optical fiber configurations to determine which provided the most uniform, fast, and stable intradermal heating. The target intradermal temperature for this experiment was 43°C, which was previously determined to be the ideal moderate heating temperature for GET both *in vitro* and *in vivo* (79,37). An intradermal temperature of 43°C was achieved after operating the infrared laser across all nine optical fibers arranged in three rows for 30 seconds at 8 amps. This elevated temperature was maintained for approximately 30 seconds, thus allowing for the maximal 20-second GET pulse protocol to be completed while the tissue remained moderately heated (Figure 9). These heating parameters and duration were in-line with previously reported results from our group utilizing the same infrared laser though here we present an updated applicator design (78). The temperature remained elevated above 41°C for 20 seconds. There was no significant difference between all tested heating configurations: three, six, or nine

optical fibers. All moderate heating configurations were repeated at three individual sites. The data reported represent the average temperature (°C) ± the standard deviation.

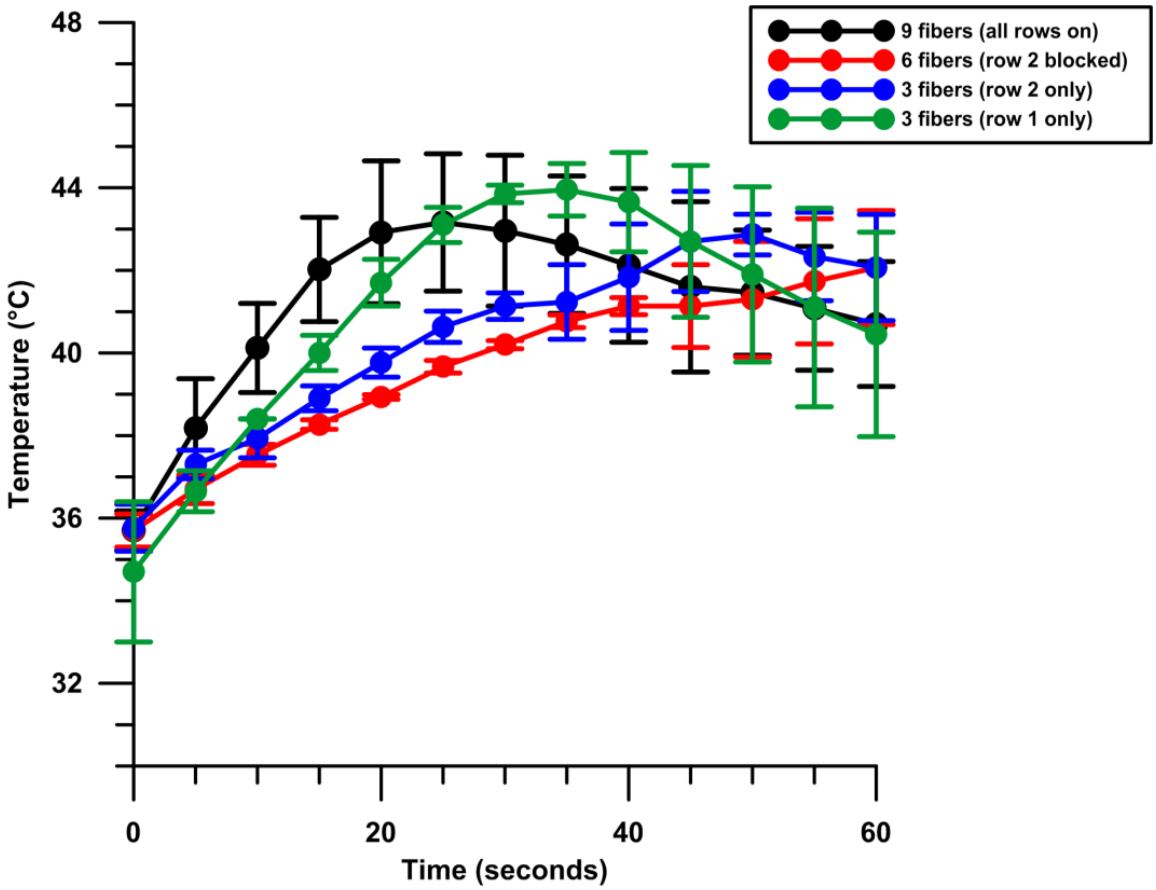

Figure 9. Intradermal skin temperature during moderate heating. *In vivo* **i**ntradermal temperature measured by a K-type thermocouple. Baseline temperature was recorded prior to each heating iteration- 3, 6, or 9 fibers. Moderate heating was applied via infrared laser positioned 1 cm away from the skin target. A target temperature of 43°C was attempted for each heating condition, after which the exogenous heating source was manually turned off and

intradermal temperature decay was measured. Temperature measurements were recorded at 5-second intervals for a total of 60 seconds. Average temperature is reported in degrees C per second ± stdev, n=3 sites per group.

CHAPTER III

DELIVERY OF REPORTER GENES USING MODERATE HEAT-ASSISTED GET

INTRODUCTION

Before advancing to a therapeutic model, the use of reporter genes is important for determining both the kinetics of expression and the distribution within the tissue following moderate heat-assisted GET. Our previous work demonstrated that expression could be enhanced and/or the applied voltage reduced by preheating the tissue prior to pulse administration. In the current study, we utilized our 16-pin MEA and incorporated nine optical fibers, connected to an infrared laser, between each set of 4 electrodes to heat the tissue to 43°C. For proof of principle, a guinea pig model was used to test delivery of reporter genes. The two reporter genes used in this work were luciferase and a DDK protein tag each encoded on a plasmid with a gWiz backbone and pCMV promoter.

While increased temperature can enhance delivery when used together with GET, it is also important to moderate the applied heat so as not to cause tissue damage. We previously established that the optimal intradermal temperature for enhancing GET was 43°C. It takes several minutes to cause a burn at 43°C, our pulse protocol is far under this threshold. We developed a moderate heating protocol using an infrared laser that demonstrated a 30 second preheating duration was sufficient for sustained temperature during the proceeding pulsing protocol. Tissue integrity following moderate heat-assisted GET was evaluated through histological analysis using an H&E staining protocol. Furthermore, a dosing study was carried out where both volume and concentration of plasmid DNA were varied to determine the optimal injection for moderate heat-assisted GET for achieving the greatest coverage and

overall expression levels. The synergy between moderate heating and electroporation allows for the reduction of both the necessary applied voltage and pulse number. These reductions have the potential to create an application that is less painful for the recipient and easier to apply for the clinician.

MATERIALS AND METHODS

Animals

Animals used for this study were 8-10-week-old Female Hartley guinea pigs weighing approximately 300-350g at the time of treatment. The animals were housed at the Old Dominion University animal facility and procedures approved by the Old Dominion Institutional Animal Care and Use Committee approval #17-022. All animal subjects were quarantined for a minimum of 7 days following arrival and prior to the conduction of any procedures.

Animal euthanasia was performed at the conclusion of each respective observation period. Euthanasia was carried out by first anesthetizing the animals in a chamber filled with 4% isoflurane combined with 96% oxygen. After 15 minutes, the animals were transferred to a separate chamber and euthanized by exposure to carbon dioxide for 15 minutes. Compressed gas was used as the carbon dioxide source. The chamber was not pre-filled, but slowly filled, and cleaned and dried between animal groups. A bilateral thoracotomy was performed to confirm euthanasia. This secondary euthanasia procedure was only performed after cessation of respiratory and cardiovascular movements was confirmed. Euthanasia procedures were approved by the IACUC at ODU and were followed in accordance with those guidelines.

Plasmids

Endotoxin-free plasmid preparations at 2mg/ml in 0.9% sterile injectable saline of gWizLuc, encoding firefly luciferase, and gWizLuc-myc-DDK, encoding the oncogene myc and a DDK protein tag, were commercially prepared for this study (Aldevron, Fargo, ND). Both plasmids have a gWiz backbone and a CMV promoter.

Infrared laser heat application

Animals were anesthetized with O_2 containing 2.5-3.0% isoflurane. Prior to treatment both flanks were shaved and washed with mild soap and water to remove loose hair and cleanse the skin of any abundance of oil. Moderate heating via a Class IV Laser power supply (LaserMate Group, Inc) via a laser optic fiber (Model: M79L005 Thor Labs Inc.) was applied immediately following a 50µL 2mg/mL intradermal injection of plasmid DNA to the pre-washed and shaven flank skin for a total duration of 30 seconds, after which the laser was switched off. All safety precautions were adhered to while the laser was in operation, including eye protection and barriers. All staff were trained and received operation approval via the Radiation & Laser Safety Program at Old Dominion University prior to the conduction of any experiments.

Reporter gene delivery

Animals were anesthetized with O_2 containing 2.5-3.0% isoflurane. Animals were pre-shaven and washed with mild soap and water to remove any loose hair or an overabundance of oil. A 100µg intradermal injection of plasmid DNA was delivered to the flank. Injection sites were marked to ensure accuracy of data collection. The electrode array was immediately positioned over the injection area with or without exogenous moderate heating. For these studies both 2 and 4 rounds of 18, 150ms pulses were delivered, yielding a total of 36 and 72

pulses respectively. In addition, applied voltages of 35V and 45V were assessed. Gene expression levels were measured for all conditions by *in vivo* bioluminescent imaging or fluorescent imaging.

In vivo bioluminescent imaging and kinetic expression analysis

On days 2, 5, 7, 9, and 14, animals were anesthetized with O_2 containing 2.5-3.0% isoflurane followed by a single subcutaneous injection of D-luciferin (Gold Biotechnology, St. Louis, MO) at 150mg/kg administered at the neck scruff. The animals were confined in an anesthesia chamber for eight minutes then transferred to the IVIS® Spectrum (Perkin Elmer, Akron, OH), imaging chamber under constant anesthesia. Regions of interest (ROI) were selected on the image to encompass the entirety of each injection site independently and compared to untreated control ROIs. After background correction, bioluminescence results were represented as average total flux in photons/sec (p/s).

Immunofluorescence staining and distribution expression analysis

The localization of gene delivery within the skin was determined by immunofluorescence staining for the DDK tag protein. Skin samples were collected 48 hours post gene transfer, fixed in 4% paraformaldehyde, paraffin embedded, and sectioned to six-micron thickness by IDEXX Laboratories, Inc. (Westbrook, Maine). Hematoxylin and eosin staining was also performed on serial sections by IDEXX Laboratories. Unstained sections were deparaffinized using CitriSolv, and rehydrated in gradient alcohol (100, 95, 75, 50, and 25%). Antigen retrieval was performed in citric acid (pH 6). Cell permeabilization was performed for 20 min in 0.25% Triton X-100 in phosphate buffered saline (PBS). Blocking was performed with 4% bovine serum albumin in phosphate buffered saline with 0.01% Tween 20 (PBST) for 1 h at

room temperature. Sections were then stained for immunoreactivity with DDK-tag protein with a mouse monoclonal anti-DDK antibody (TA50011–1, OriGene, Rockville, MD) diluted 1:200 in blocking buffer overnight at 4 °C. Samples were then labeled with an AlexaFluor488 conjugated goat anti-mouse IgG secondary antibody (ThermoFisher Scientific, Grand Island NY).

All samples were washed with PBST five times for three minutes each time on a shaker between antibody applications. Negative control samples were treated with secondary antibody only, without the primary antibody. All staining procedures were performed in the dark to prevent photobleaching. Samples were counterstained with DAPI for cell nuclei identification and mounted with VECTASHIELD® HardSet™ mounting medium (Vector Laboratories, Burlingame, CA) after which they were set at room temperature in the dark overnight. After the coverslips had set, the samples were stored at −20 °C until imaging.

Plasmid dosing for moderate heat-assisted GET

Volume and concentration of plasmid DNA was assessed to determine the appropriate dose to achieve the highest expression level with minimum adverse tissue effects. For this experiment we used a plasmid encoding the reporter gene luciferase. Anesthesia was performed by placing the animals in an induction chamber infused with 3-4% isofluorane and 96-97% oxygen gas. After animals were sufficiently anesthetized, they were fitted with a standard rodent mask supplied with 3% isofluorane and oxygen to maintain a surgical plane of anesthesia. Animals were placed on a heating pad to ensure thermoregulation during the entire procedure. Animals were shaven and washed with mild soap (Dial) and water in an outward circular motion to remove any loose hair or an overabundance of oil.

Injection sites were marked to ensure accuracy of data collection. gWizLuc plasmid doses included volumes of 50 or 100µL at 0.5, 1, or 2mg/mL. The electrode array was immediately positioned over the injection area. When applicable, moderate heating was always applied after injection, but before GET. The four experimental groups included injection only (IO), injection of pDNA followed by 72 pulses at 45V (45V 72 p), injection of pDNA followed by 72 pulses at 35V with moderate heat (35V 72p + heat), and injection of pDNA followed by 36 pulses at 45V with moderate heat (45V 36p + heat). Each experimental group was tested after injection of the respective plasmid concentration and volume. Gene expression levels were measured 2, 5, 7, 9, and 14 days after gene delivery via *in vivo* bioluminescence imaging.

Statistical analysis

Statistical significance between groups for the luciferase reporter gene experiment was determined using a 2-way analysis of variance (ANOVA) with a Tukey-Kramer multiple comparisons posttest. Results are expressed as the mean of 5-8 replicates per group (± SEM). Significant results were determined with respect to animals receiving injection of plasmid DNA alone unless otherwise noted. A *P* value less than 0.05 was considered significant.

Statistical significance between groups for the reporter gene dosing experiment was determined using a 2-way analysis of variance (ANOVA) with a Tukey-Kramer multiple comparisons post-test (GraphPad Prism Software, La Jolla, California). Results are expressed as the mean of 4-8 replicates per group (± SEM). Significant results were determined with respect to animals receiving injection of plasmid DNA alone unless otherwise noted. A *P* value less than 0.05 was considered significant.

RESULTS

Moderate heat-assisted GET yields sustained expression levels compared to unheated counterpart

Moderate heat-assisted GET was demonstrated in a guinea pig model using the reporter gene firefly luciferase as a preliminary tool to test gene delivery in the skin. The luciferase expression pattern following the application of various pulsing parameters with and without the addition of moderate heat was recorded over a two-week observation period (Figure 10**A**). Peak expression *in vivo* was observed 48 hours after exposure in all tested conditions. The highest level of expression, 3.66×10^8 photons/second (p/s), was achieved with 45V 36 pulses plus moderate heat at this time point ($p<0.05$). Furthermore, this same condition yielded the highest expression levels at both observation days 5 and 7 with a gradual decline over the 14-day observation period ($p<0.01$). Results indicate that the pulse number and applied voltage could be reduced by 50% (72 to 36 pulses) and 23% (45V to 35V) respectively, with the addition of moderate heating via infrared laser to achieve the same expression levels.

We also determined the effect of reducing the number of active optical fibers for applying moderate heat to the target. As described, the infrared laser-heating component is distributed equally among nine optical fibers that are arranged equidistant in spans of three across three rows. They can be blocked individually or by row, therefore allowing for sharp control of the thermal distribution. We tested the efficacy of moderate heat row by row in combination with our highest performing GET condition- 45V 36 pulses. There was no significant difference between applying moderate heat with 3, 6, or 9 optical fibers in our aforementioned highest expressing GET protocol (Figure 10**B**).

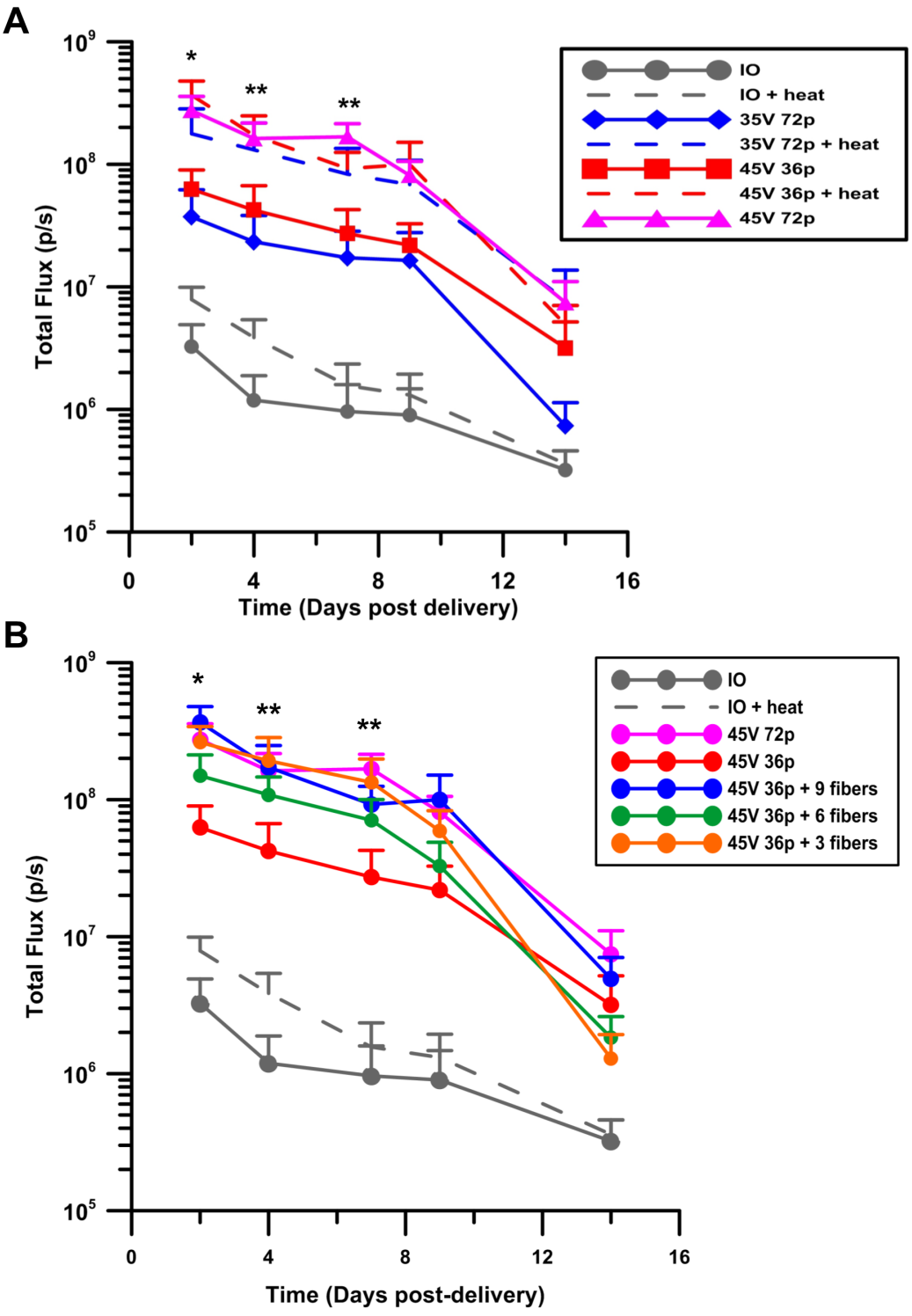

Figure 10. Expression kinetics of moderate heat-assisted GET to the skin. (A) Experimental groups included injection only (IO) plus heat (IO+heat), injection of pDNA followed by 72 pulses

at 45V (45V 72p) or 35V (35V 72p), 72 pulses at 35V with moderate heat (35V 72p + heat), and 36 pulses at 45V with (45V 36p + heat) or without heating (45V 36p). (B) Flexibility and distribution of heating was assessed for 3, 6, and 9 active optical fibers followed by GET at 45V 36p. Luciferase expression levels reported as average total flux (photons/second) ± SEM, n=8 individual sites per group. *****, *p<0.05*, ******, *p<0.01*.

Gene expression following moderate heat-assisted GET extended to the dermis and underlying muscle

To determine the distribution and localization of gene delivery following moderate heat-assisted gene electrotransfer, fluorescence staining was performed. The reporter gene, gWizLuc-myc-DDK, was delivered to guinea pig skin via intradermal injection with or without GET and moderate heat (Figure 11). No expression was observed following an intradermal injection with moderate heat alone (Figure 11**A**). Immunohistochemical evaluation demonstrated that expression was localized in the dermis and muscle layer of the tissue in those animals receiving moderate heat-assisted GET (Figure 11**F-G**). The addition of moderate heat resulted in a higher level of expression at the reduced 35V compared to the same condition treated at room temperature (Figure 11**B-C**). When GET was combined with moderate heat using half the amount of pulses, 36 (Figure 11**F-G**) versus 72 (Figure 11**D-E**), this expression pattern was more pronounced suggesting a long-lasting effect.

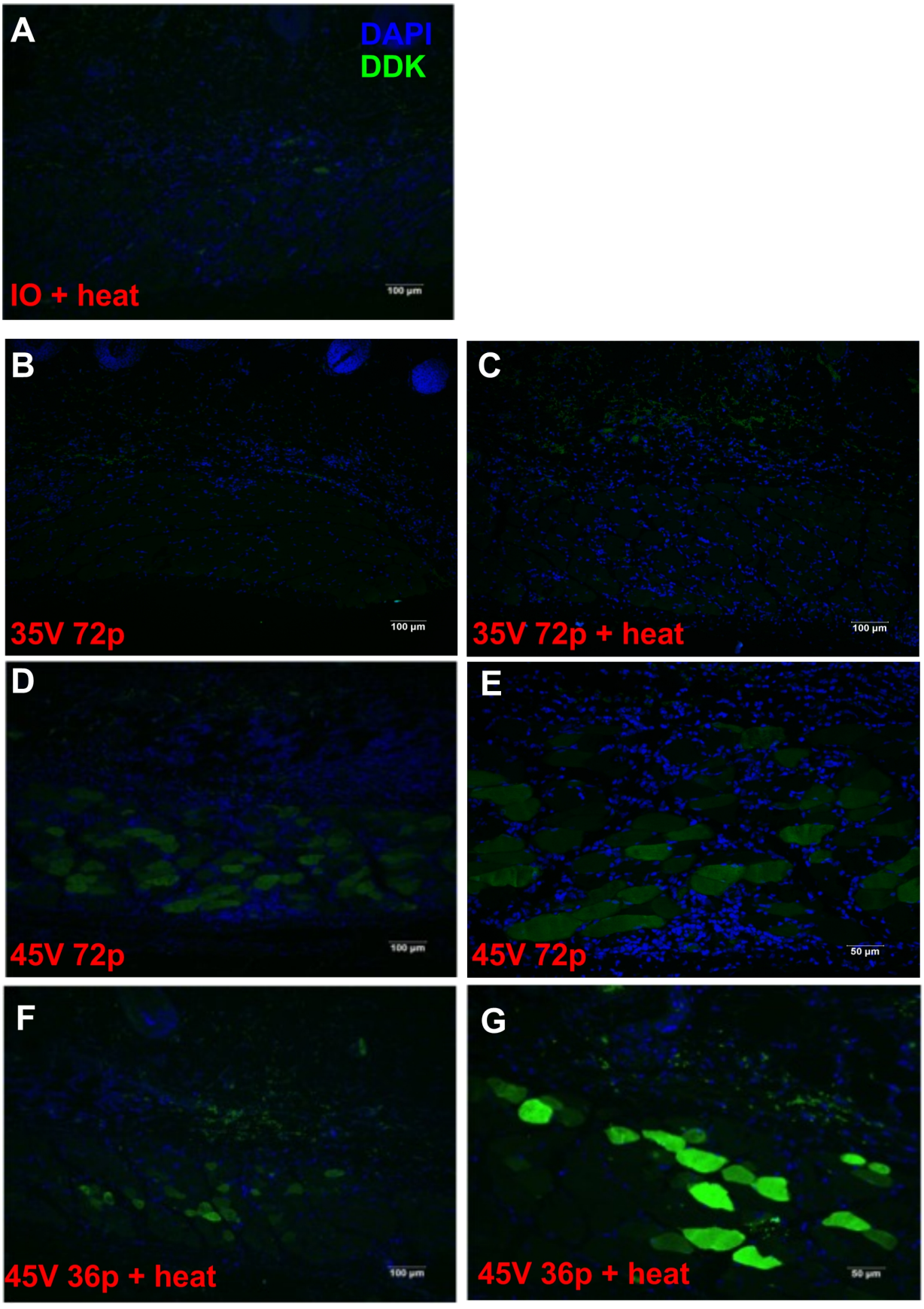
A
DAPI
DDK
IO + heat
100 µm
B
35V 72p
100 µm
C
35V 72p + heat
100 µm
D
45V 72p
100 µm
E
45V 72p
50 µm
F
45V 36p + heat
100 µm
G
45V 36p + heat
50 µm

Figure 11. Distribution of expression following moderate heat-assisted GET to the skin. An indirect fluorescence staining protocol using an anti-DDK primary antibody and secondary antibody, Alexa Fluor 488, was performed on guinea pig skin fixed in 4% PFA 48 hours after GET. Samples were paraffin embedded and sections were counterstained with DAPI. Images are representative of 3-4 individual subjects. Experimental groups included (A) injection only plus heat (IO+heat), (B,C) injection of pDNA followed by 72 pulses at 35V at ambient (35V 72p) or after the application of moderate heating (35V 72p +heat), (D,E) 72 pulses at 45V (45V 72p) applied at ambient, and (F,G) 36 pulses at 45V after the application of moderate heat from an infrared laser (45V 36p + heat). (A-D, F) 100x, scale bar =100μm. (E,G) 200x, scale bar = 50μm.

Moderate heating mitigates a reduction in pulse number and skin damage caused by high voltage GET

Histological analysis was performed to assess tissue damage after the application of GET (Figure 12). H&E staining on fixed tissue sections revealed that the application of a higher pulse number at a higher applied electric field resulted in a burn extending from the epidermis to the hypodermis with infiltrating cells suggesting injury (Figure 12**A, C**). When moderate heating was applied at the same electric field at 50% of the pulsing dose, histological damage was not observed (Figure 12**B, D**).

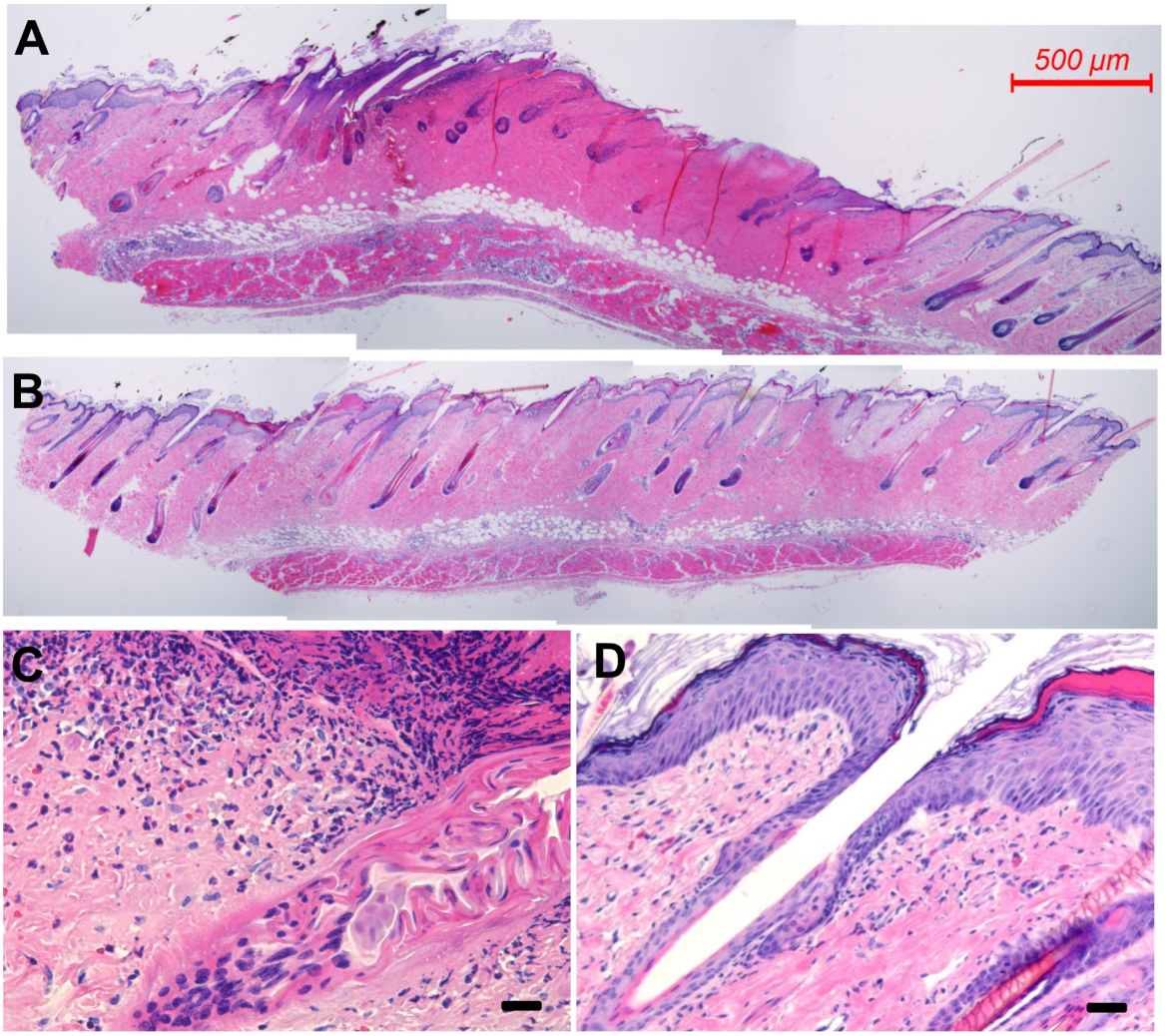

Figure 12. Histological analysis after cutaneous moderate heat-assisted GET. H&E staining was performed on guinea pig skin fixed in 4% PFA and paraffin embedded. Images are representative of 3-4 individual subjects. Experimental groups included (A) injection of pDNA followed by 72 pulses at 45V (45V 72 p no heat) and (B) 36 pulses at 45V after the application of moderate heat from an infrared laser (45V 36p + heat). 40x, scale bar = 500µm. (C) Cell infiltrate visible at site of damage following 45V 72 pulses, 200x, scale bar = 10µm. (D) Intact tissue with no apparent damage after 45V 36p + heat, 200x, scale bar = 10µm.

Increasing the volume of plasmid DNA and not the concentration yielded highest expression levels

Plasmid DNA dosing was optimized by varying both the concentration and intradermal injection volume at each delivery site. For this work, the reporter gene luciferase was used to determine the kinetics of luciferase expression following the application of various pulsing parameters with and without the addition of moderate heat. Two volumes: 50 and 100µL and three concentrations: 0.5, 1, and 2mg/mL were evaluated across four experimental conditions including injection only, moderately heated GET conditions of 36 pulses at 45V and 72 pulses at 35V, and ambient GET conditions of 72 pulses at 45V.

Peak luciferase expression *in vivo* was observed 48 hours after exposure in all tested parameters and decreased gradually over the two-week period of observation (Figure 13). A 100µL injection at 1mg/mL yielded the highest overall level of luciferase expression in all tested parameters compared to all other concentrations and volumes. The highest expression was shown in those animals receiving 36 pulses at 45V with a total flux of $9.9x10^8 \pm 1.8x10^8$ photons/sec after just two days (Figure 13**C**). These results were significant compared to injection of plasmid DNA alone (*p<0.001*) (Figure 13**A**). Similarly, 72 pulses at 45V delivered at ambient temperature following a 100µL injection at 1mg/mL resulted in a total flux of $8.84x10^8 \pm 5.66x10^8$ photons/sec (Figure 13**D**). However, this condition served as a positive control, in that although expression levels were high, the superficial damage attributed to these GET parameters were unfavorable and negated its potential use for translation.

In the high-pulse, low-voltage moderately heated GET condition (35V 72p +heat), the highest expression level was also observed at day 2 with a total flux of $2.38x10^8 \pm 2.82x10^7$

photons/sec after a 100µL injection at 1mg/mL (Figure 13**B**). Though at a peak level lower than that of the other tested GET conditions, these results were significant with respect to those animals receiving injection of plasmid DNA alone of the same volume and concentration (*p<0.01*). Though not significant, on observation days 7 and 9, animals receiving the higher plasmid DNA concentration of 2mg/mL in an injection volume of 100µL followed by 35V 72 pulses plus moderate heating, displayed a trend of elevated expression levels compared to the other five dosing parameters at the same time point.

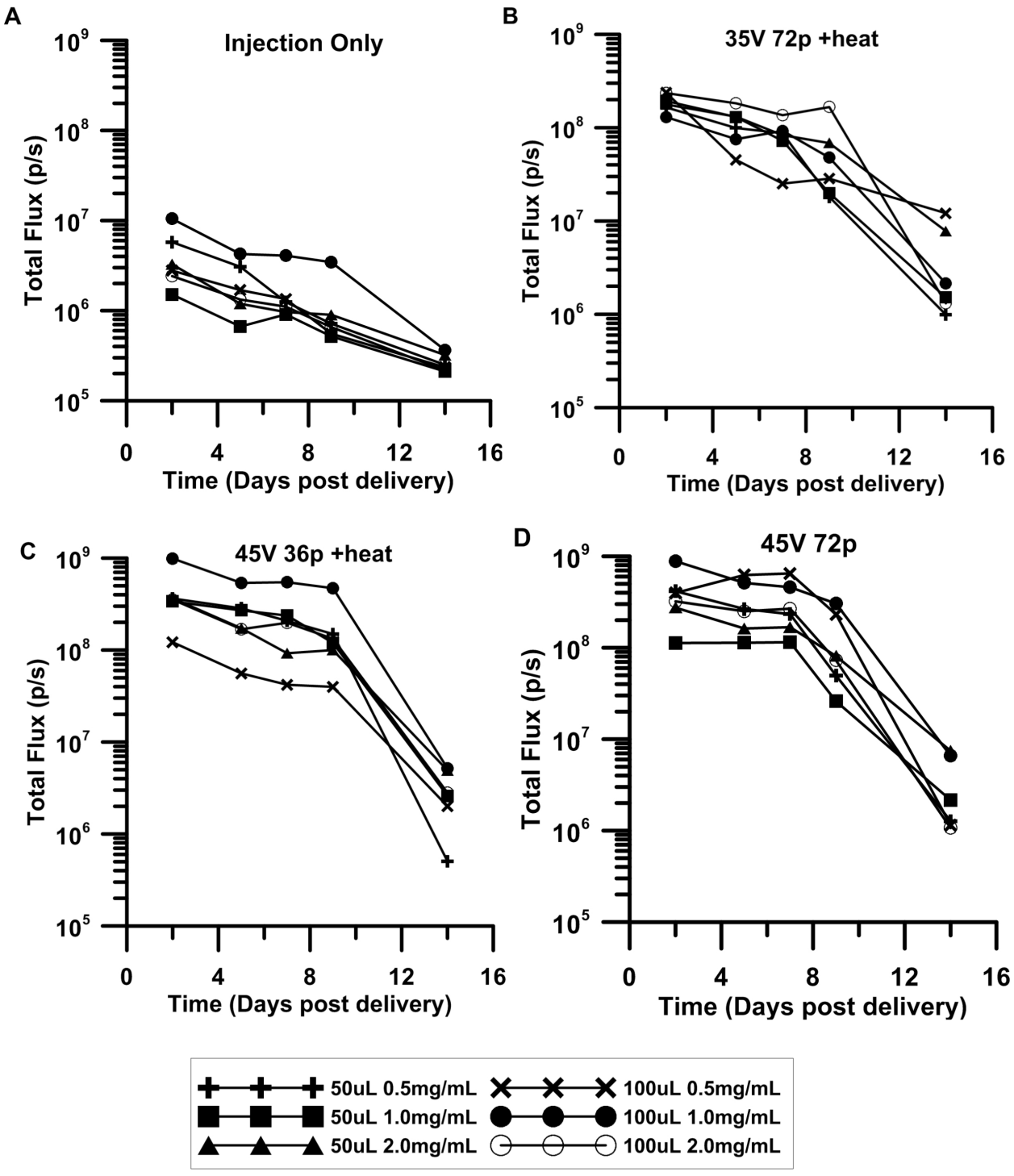

Figure 13. Optimization of plasmid DNA dosing for moderate heat-assisted GET to the skin. Experimental groups included (A) injection pDNA only (IO), (B) 72 pulses at 35V with moderate heat (35V 72p + heat), injection of pDNA followed by GET with (C) 36 pulses at 45V with

moderate heating (45V 36p + heat) or (D) 72 pulses at 45V without moderate heating (45V 72 p). Plasmid DNA doses included volumes of 50 or 100µL at 0.5, 1, or 2mg/mL. Luciferase expression levels reported as average total flux (photons/second), n=5-8 individual sites per group.

DISCUSSION

There are a number of associated effects of thermal application to consider including pain sensation, skin discoloration, inhibition of cellular processes, and injury. In the current work, we present a platform for reversible electroporation in the skin, where cell viability is maintained. We heat the skin to a moderate temperature of 43°C for a total of 30 seconds and maintain an elevated temperature for an additional 20 seconds after the exogenous heat source is removed. Our previous work in an *ex vivo* pig skin model and in a 3D human skin model, suggested that moderate heating via an infrared laser provided fast, uniform heating of the target. At this temperature, the skin is temporarily warm and normal metabolism still proceeds. We observed no injury to the skin after moderate heat was applied *in vivo*. Conversely, high voltages and pulse number led to skin damage both macroscopically and microscopically. We observed this effect by way of reddened skin and confirmed through H&E, in animals receiving 45V and 72 pulses without moderate heat, suggesting that although this condition yielded a high level of expression, it was damaging and not ideal for translation for non-life-threatening applications. The combination of moderate heating and GET allowed for a significant reduction in both pulse number (50%) and applied electric field (23%) all while

achieving similar, or even higher overall luciferase expression levels in some cases. Moreover, tissue damage was not observed in reduced GET conditions with moderate heating. These are promising features of moderate heat-assisted GET, providing a shorter, less painful delivery platform compared to high voltage-high pulse GET performed at ambient. The result is a faster, minimally invasive route of electrotransfer.

We observed expression in the lower dermis and muscle following moderate heat-assisted GET. These data are encouraging as the propensity for a deliverable to reach systemic circulation is higher in these tissues. Furthermore, our data demonstrate that an intradermal approach can yield expression in the muscle using a non-penetrating electrode with the addition of moderate heating. The enhanced membrane fluidity associated with temperature increases could explain this phenomenon. Interestingly, we observed little expression in the epidermis, the local site of delivery, suggesting the plasmid DNA is electrophoretically pushed by both the applied electric field and the application of moderate heat.

Lastly, we sought to optimize the volume and concentration of plasmid to achieve the highest level of transgene expression. We found that a 100µL injection at 1mg/mL achieved the highest level of luciferase expression when followed by 45V 36 pulses with moderate heating. This is likely attributed to better coverage of the plasmid DNA injection by the applied electric field and exogenous moderate heating source, given that a 100µL injection measures approximately 8mm, while a 50µL spans approximately 5mm. Taken together these results prompted future studies on the utility of moderate heat-assisted GET using therapeutic agents.

CHAPTER IV

MODERATE HEAT-ASSISTED GET FOR INTRADERMAL DNA VACCINCATION AGAINST HEPATITIS B VIRUS

INTRODUCTION

Hepatitis B virus (HBV) is a leading global cause of liver disease (83-86). HBV infection is endemic in Asia, the Pacific Islands, Eastern Europe, and Sub-Saharan Africa where most of the population are carriers (87-89). The virus is transmitted via blood or sexual contact and is quite resilient, able to survive outside the body for up to seven days. Each year there are approximately 200,000 new HBV cases in the US (90). The symptoms of HBV infection are non-specific and infection is generally confirmed through blood laboratory analysis once latent indications appear. Preventative HBV vaccination has reduced the disease burden; however, an estimated 350 million people worldwide are living with chronic HBV (91).

Vaccination schedule compliance is critical for HBV prevention. Current guidelines recommend beginning vaccine dosing within 24 hours of birth, followed by two to three additional doses within the first year of life (92,93). Global vaccination estimates of children are unclear on the extent to which vaccines are administered completely and on time. In practice, vaccines tend to be given later rather than earlier. When HBV vaccination is delayed, children fail to receive adequate protection when they are most vulnerable. Late vaccinations raise the risk of HBV infection by lengthening the period of susceptibility. This can have important implications in countries where HBV infection is endemic, increasing the probability of transmission. In this situation, catch-up vaccination of older children has relatively little impact for prophylaxis because infection may already have been acquired by the time they present for vaccination. Vaccine compliance in endemic regions is related to access barriers. Limited

facilities, inadequately funded vaccination programs, means and distance of travel to clinics, storage logistics and capacity all hinder immunization coverage. The current recommended HBV vaccine is recombinant protein consisting of the viral envelope protein Hepatitis B surface antigen and requires strict cold storage to maintain its stability (83,90-92).

DNA vaccines are an attractive alternative to standard vaccine types, given their innocuous make-up and simplicity of production requirements, however there are several pitfalls. Previous DNA vaccines in the clinical pipeline have demonstrated poor cellular uptake and immunogenicity rendering them inconsistent or ineffective for conferring protection. These shortcomings can be mediated through enhanced delivery protocols beyond the standard needle-fitted syringe administration. Delivery of plasmid encoding viral antigen through GET has potential as a DNA vaccination platform (38,40,94-97). A recent preclinical study delivering a DNA vaccine via GET showed HBV-specific T-cell responses in humans (97,98).

Here we present moderate heat-assisted GET as a novel vaccine delivery platform utilizing a combination of moderate heating and non-invasive electrotransfer via the MEA. We have previously shown the advantages of combining electrotransfer with moderate heating where both pulse number and applied voltage could be significantly reduced resulting in a shorter and less painful method (37,78). Futhermore, using the MEA for intradermal delivery has been shown to be both effective and tolerable (39,78,80,81,99). Minimizing discomfort is an important consideration for translation to non-life-threatening applications such as vaccination, in which schedule compliance is critical to achieve immunization coverage. Here we present moderate heat-assisted GET for the delivery of a DNA vaccine against HBV. To evaluate this method, we utilized a guinea pig model for delivery of a DNA vaccine encoding

Hepatitis B surface antigen. The objective of this work was to determine the appropriate moderate heat-assisted GET parameters for achieving elevated Hepatitis B surface antibodies in circulation with the overarching goal of conferring humoral immunity against HBV.

MATERIALS AND METHODS

Animals

Animals used for the HBV vaccination study were 8-10-week-old Female Hartley guinea pigs weighing approximately 300-350g at the time of treatment. The animals were housed at the Old Dominion University animal facility and procedures approved by the Old Dominion Institutional Animal Care and Use Committee approval #17-022. All animal subjects were quarantined for a minimum of 7 days following arrival and prior to the conduction of any procedures.

Animal euthanasia was performed at the conclusion of the 30-week observation period. This procedure was previously described in Chapter III and was performed in the same manner for this study. Briefly, Euthanasia was carried out by first anesthetizing the animals in a chamber filled with 4% isoflurane combined with 96% oxygen. After 15 minutes, the animals were transferred to a separate chamber and euthanized by exposure to carbon dioxide for 15 minutes. Compressed gas was used as the carbon dioxide source. The chamber was not pre-filled, but slowly filled, and cleaned and dried between animal groups. A bilateral thoracotomy was performed to confirm euthanasia. This secondary euthanasia procedure was only performed after cessation of respiratory and cardiovascular movements was confirmed. Euthanasia procedure were approved by the IACUC at ODU and followed in accordance.

Infrared Laser system for moderate heating

For moderate heat application, the electrode was placed with the spring-loaded GET electrode pins in contact with the skin, leaving a fixed distance of 1cm between the optical fibers and the vaccine target. The laser was applied for a total of 30 seconds following injection of plasmid DNA. This duration allowed for 43°C to be maintained for 30 seconds after removal of the moderate heating source. The GET protocol was approximately 20 seconds in duration, enabling the tissue to remain heated during its application. Moderate heating was always applied prior to and never concurrently with GET. Safety precautions such as eyewear and barriers were in place during all laser operations. All staff were trained and received operation approval via the Radiation & Laser Safety Program at Old Dominion University prior to the conduction of any experiments.

GET operating parameters

The moderate heating and GET delivery was incased in a single device. The design of the MEA GET delivery device has been previously described (80,81,99). Briefly, the electrode has 16 pins arranged in a 4x4 pattern at 2-mm-apart. Each pair of electrodes was programmed to administer either a train of four pulses with total 72 pulses or two pulses for a total of 36 pulses depending on pulsing conditions. The applied voltage was either 35 or 45 V between two conductive pins, each with a pulse duration of 150 ms and a 150 ms delay between pulses. Electrotransfer was performed using the UltraVolt Model: Rack-2-500-00230 (UltraVolt, Inc. Ronkonkoma, NY, USA).

Plasmids

For the HBV vaccination study, endotoxin-free plasmid encoding Hepatitis B surface antigen (gWizHBsAg) was professionally prepared and suspended to 2 mg/mL in 0.9% sterile injectable saline (Aldevron). The plasmid has a gWiz backbone and a pCMV promoter.

Moderate heat-assisted GET delivery of pHBsAg

Female guinea pigs weighing approximately 350 g were used for this study as they represent a model for human skin. Anesthesia was performed as previously described. After animals were sufficiently anesthetized, they were fitted with a standard rodent mask supplied with 3% isofluorane and 97% oxygen to maintain a surgical plane of anesthesia. The right flank was shaved and washed in the same aforementioned fashion, ensuring the removal of sebaceous oils prior to GET. Moderate heat-assisted GET was applied after the preoperative cleansing procedure. The five tested groups included: IO + heat (injection of plasmid without GET and with moderate heating), pHBsAg+45V 36p (plasmid with high-voltage low-pulse GET), pHBsAg+45V 36p+heat (plasmid with moderate heating and high-voltage low-pulse GET), pHBsAg+35V 72p (plasmid with moderate heating and low-voltage high-pulse GET) and pHBsAg+45V 72p (plasmid with high-voltage high-pulse GET). pHBsAg was injected at a single site intradermally in a 100 µL volume at a concentration of 1mg/mL (100 µg total) immediately before moderate heating via infrared laser or GET application. Operating parameters for both moderate heating and GET were delivered as previously described. After primary vaccination, each animal was monitored continuously until they recovered from anesthesia, as indicated by their ability to maintain sternal recumbency and exhibit purposeful movement. Boost

vaccinations were administered 14 days after the prime protocol in appropriate subjects in the same manner, except on a different location on the animal's flank.

HBsAg neutralizing antibody production

An ELISA kit specific for guinea pig anti-HBsAg (KA0286, Novus Biologicals) was used to detect anti-HBs in all animal subjects. Sera was collected via a jugular vein puncture and isolated in serum separator tubes, which were then assayed per the manufacturer's instructions. Serum was collected 6, 11, 18, 24, and 30 weeks after immunization. Sera collected prior to vaccination was used to determine baseline levels of circulating anti-HBs. Two standard deviations from the mean of this baseline served as a positive result. Serial dilutions were performed on the serum samples to accommodate these criteria. The results are reported as HBsAb Geometric Mean Titer.

Statistical Analysis

Statistical significance between the groups for HBV DNA vaccination was determined by 2-way ANOVA with a Tukey-Kramer multiple comparisons post-test (GraphPad Prism Software, La Jolla, California). Results are expressed as the mean of 5 individuals per group (± SEM). A *P* value less than 0.05 was considered significant.

RESULTS

Moderate heat-assisted and ambient GET induces the production of antibodies against HBV surface antigen following intradermal injection of pHBsAg

Circulating antibodies specific to HBV surface antigen have been shown to be efficacious for conferring HBV humoral immunity. Surveillance of HBsAb titer is used clinically to determine if a follow-up boost vaccination is warranted, as a drop in levels of this antibody suggests a drop

in immunization protection. The ability of moderate heat-assisted GET to induce the production of HBsAbs after injection of a plasmid encoding HBV surface antigen was tested by comparing the levels of antibody titers in the serum to unheated GET conditions. All GET parameters had been previously tested for gene expression in the dosing optimization experiment. Based on results from the dosing experiment, an intradermal injection of 100μL at 1mg/mL was used for this work. GET parameters for DNA vaccination against HBV included: injection only plus moderate heating, 45V 36 pulses with and without moderate heating, 35V 72 pulses plus moderate heating, and 45V 72 pulses without moderate heating. Guinea pigs were injected with pHBsAg followed by appropriate GET and/or moderate heating conditions. A boost vaccination was given in the same manner after 2 weeks at a different site than the prime. Serum collected via the jugular vein over a 30-week observation period was assayed using an ELISA.

Overall, HBsAb titers induced by DNA vaccination with pHBsAg via all GET parameters, moderately heated or ambient, were significantly higher than an injection only control with moderate heating. All GET groups displayed a steady increase in antibody production, peaking at week 18 and gradually waning out to the end of the 30-week observation period. Notably, 45V 36 pulses with moderate heating yielded significantly higher HBsAb titers compared to all other tested conditions at all observation time points ($p<0.001$) (Figure 14). By week 18, HBsAb titers in this condition (45V 36p +heat) peaked and were 230-fold higher than those in an injection only control with moderate heating (IO +heat) ($p<0.001$). Furthermore, antibody production at week 18 in the experimental condition 45V 36 pulses with moderate heating were 20-fold over the HBsAb titer achieved by its ambient counterpart (45V 36p) ($p<0.01$).

Though the plasmid dosing experiment carried out with the luciferase reporter gene indicated that expression levels following GET with 45V 72 pulses were similar to 45V 36 pulses plus moderate heating, we did not observe such similarities in HBsAb antibody production between these two treatments. Specifically, at peak antibody production, 45V 36 pulses with moderate heating was still nearly 10-fold higher than the levels achieved with 45V 72 pulses delivered at ambient temperature (*p<0.01*).

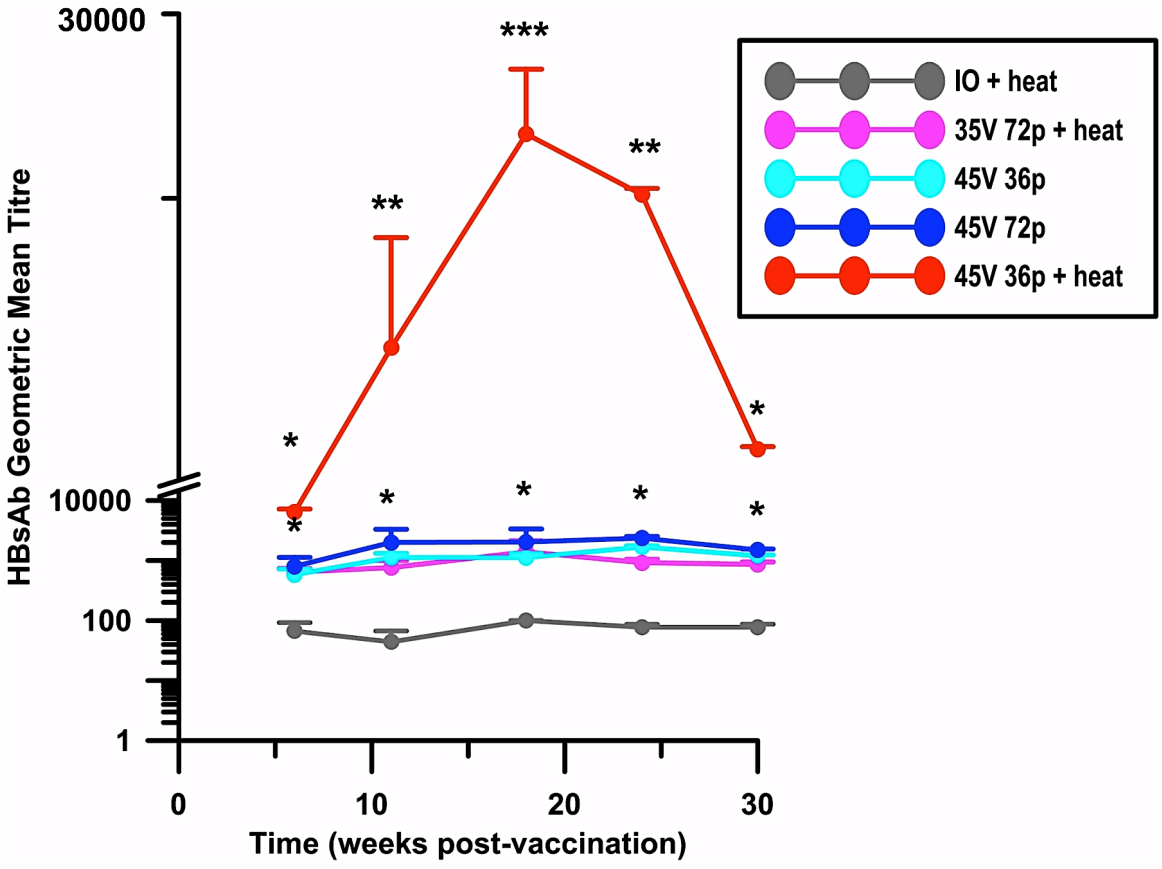

Figure 14. HBsAb production induced by moderate heat-assisted GET after a prime-boost DNA vaccination protocol against HBV. Prime and boost vaccinations were separated by two weeks.

All animals received an intradermal injection of a plasmid encoding Hepatitis B surface antigen (pHBsAg) in a 100µL volume at 1mg/mL. Experimental groups included injection pDNA only plus moderate heating (IO+heat), injection of pDNA followed by GET with 72 pulses at 45V (45V 72 p), 72 pulses at 35V with moderate heat (35V 72p + heat), and 36 pulses at 45V with (45V 36p + heat) or without moderate heating (45V 36p). Serum was collected via jugular vein puncture 6, 11, 18, 24, and 30 weeks after immunization. Serum collected prior to vaccination was used to determine baseline levels of circulating HBsAbs. HBsAb titer in serum was measured by an ELISA. Two standard deviations from the mean of this baseline served as a positive result. Serial dilutions were performed on the serum samples to accommodate these criteria. The results are reported as HBsAb Geometric Mean Titer ± SEM, n=5 individuals per group. *****, *p<0.05,* ******, *p<0.01,* *******, *p<0.001*,.

Moderate heat-assisted GET elicits significantly higher HBsAbs after both prime and boost vaccination schedules

HBV DNA vaccination was evaluated after a single primary dose to determine HBsAg antibody production at a reduced schedule. For this work, we assessed efficacy of the four previously tested GET groups: 35V 72 pulses with moderate heating, 45V 72 pulses, and 45V 36 pulses with and without moderate heating. After the prime vaccination protocol, peak antibody production was observed at week 6 across all tested GET parameters. Just as we observed after the boost vaccination, HBsAb titers in those animals receiving pHBsAg followed by the high-

voltage low-pulse moderately heated condition (45V 36p +heat), were significantly higher than all other GET conditions (Figure 15). At week 6, HBsAb titers were 3-fold higher in the experimental condition 45V 36 pulses with moderate heating compared to its ambient counterpart (45V 36p) ($p<0.05$). Similarly, this condition was 1.73 and 2.5-fold higher than 45V 72 pulses without moderate heating and 35V 72 pulses plus moderate heating respectively.

Overall, antibody production from the prime protocol was far lower than those levels achieved from two doses: a prime and boost given two weeks apart. However, though a lower level of antibody production was observed, the same trends between the tested GET parameters were replicated in this experiment where 45V 36 pulses plus moderate heat induced the highest overall level of HBsAb production.

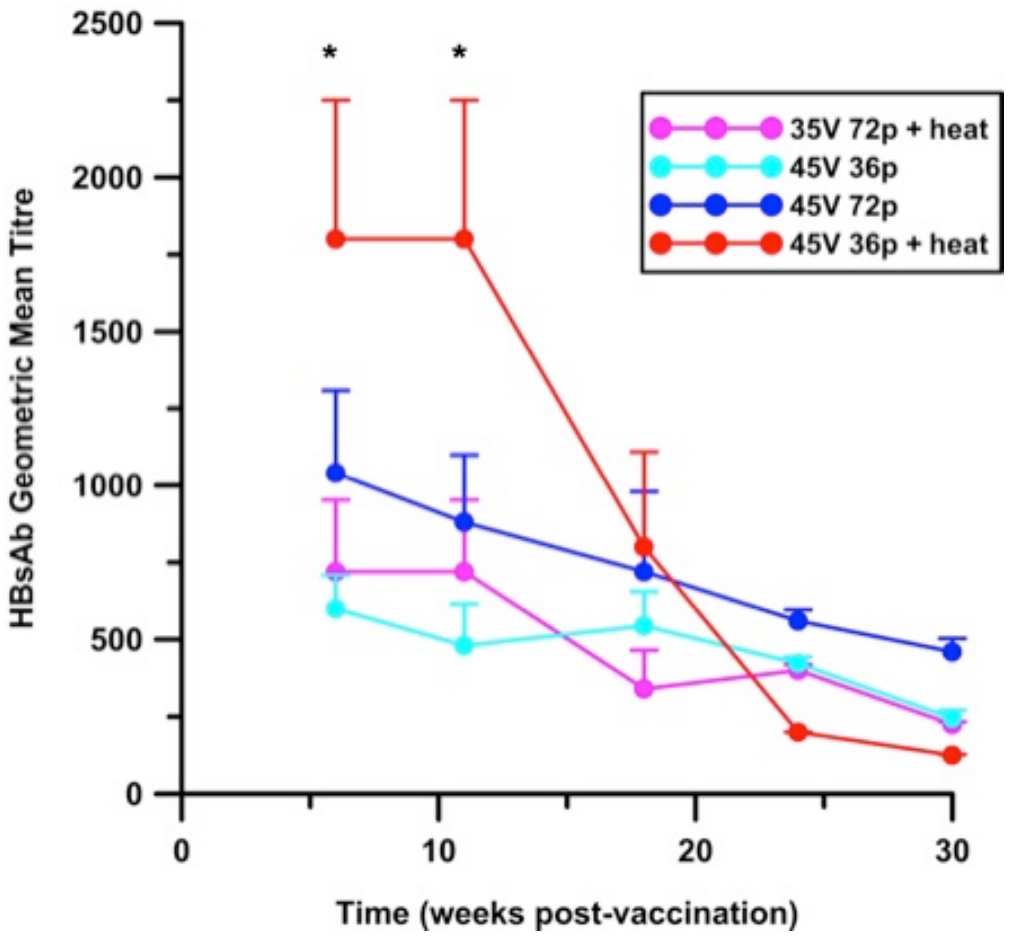

Figure 15. HBsAb production induced by moderate heat-assisted GET after a prime only DNA vaccination protocol against HBV. No boost vaccination was administered in this experiment. All animals received an intradermal injection of a plasmid encoding Hepatitis B surface antigen (pHBsAg) in a 100µL volume at 1mg/mL. Experimental injection of pDNA followed by GET with 72 pulses at 45V (45V 72 p), 72 pulses at 35V with moderate heat (35V 72p + heat), and 36 pulses at 45V with (45V 36p + heat) or without moderate heating (45V 36p). Serum was collected via jugular vein puncture 6, 11, 18, 24, and 30 weeks after immunization. Serum collected prior to vaccination was used to determine baseline levels of circulating HBsAbs. HBsAb titer in serum was measured by ELISA. Two standard deviations from the mean of this

baseline served as a positive result. Serial dilutions were performed on the serum samples to accommodate these criteria. The results are reported as HBsAb Geometric Mean Titer ± SEM, n=5 individuals per group. *****, $p<0.05$.

DISCUSSION

Whole-pathogen and subunit vaccines predominate current vaccine schedules (100). Whole-pathogen vaccines contain either killed or live-attenuated or weakened strains that cannot cause disease, but are able to elicit an immune response. Subunit vaccines are comprised of only the essential antigens of a particular pathogen along with adjuvants to achieve long-term immunity, as antigen alone is often not enough to confer protection. Though effective, these formulations require strict cold storage to maintain stability, are costly to manufacture, require multiple scheduled doses to achieve life-long protection, and can have serious side effects in some recipients, such as those with allergies or compromised immune systems, and in rare cases can lead to reversion to a disease-causing state (100-102).

DNA vaccines consist of a plasmid that carries genes encoding proteins from the pathogen of interest (95-97). When delivered, the plasmid enters host cells and serves as a genetic template for the translation of its antigen. DNA vaccines are non-live and non-replicating, leaving little risk for secondary infection from vaccination. Due to well-established production protocols, DNA plasmid vaccines can be made rapidly, shortening the time between an outbreak and the public health response. This streamlined approach is advantageous with the emergence of new pathogens. DNA vaccines are an ideal global vaccination candidate due

to their robust temperature stability, specificity to target antigen, and ease of large-scale manufacturing. These advantages make DNA vaccines an inexpensive option and therefore more feasible in low-income regions where access to the general population is a barrier (100,103,104).

Reversible electroporation, where the cells survive, is largely considered a nonthermal process. GET falls under this category where a high voltage pulse is applied creating transient permeations in the cell membrane that are resealed once the pulse is removed (105). Physical methods of GET are used both *in vitro* and *in vivo* and are well tolerated in living tissues (11,15,16,37,40,80). By adding moderate heating to this approach, there is an increase in membrane fluidity allowing for the widening of the transient permeations already caused by GET (76,106,107). Thus, the interaction of moderate heating and GET affords both a reduction in the necessary pulse number and applied voltage to achieve a similar or enhanced result (37,78). In practice, moderate heat-assisted GET provides a faster and less painful delivery platform with all the same benefits as ambient GET.

In the current work, we evaluated the use of moderate heat-assisted GET for the delivery of a DNA vaccine against HBV. Our initial testing to confirm plasmid dosing allowed for more prudence in determining the proper volume and concentration of plasmid to deliver in our vaccination study. We found that a 100µL injection at 1mg/mL achieved the highest level of luciferase expression when followed by 45V 36 pulses with moderate heating. This is likely attributed to better coverage of the plasmid DNA injection by the applied electric field and exogenous moderate heating source, given that a 100µL injection measures approximately 8mm, while a 50µL spans approximately 5mm. Previous studies using reporter genes have

determined that a total concentration of 100μg achieved the highest expression levels (37,39,78,80,82). Our results are in line with these data.

In all of our experiments we chose to use 45V 72 pulses as a positive control, which in our experience is a MEA condition that reliably induces high expression levels. However, we hypothesized this high-voltage high-pulse condition would not be suitable for translation due to observable skin damage caused from its application. On this basis, we sought to reduce either the pulse number and/or the applied voltage with the addition of moderate heat to compensate for expression loses all while maintaining tissue health. Tissue health is critical for reversible electroporation approaches such as GET, where live cells are needed to achieve gene expression in the host. Furthermore, in non-life-threatening applications such as vaccination visible scarring and pain at the delivery site are unappealing side effects. These could be overlooked in some cases, where the benefit outweighs the detriment of the side effect, but it is not ideal. We found that with the addition of moderate heat, we were able to reduce the applied voltage by 23% and the pulse number by 50% all while achieving a similar luciferase expression level and eliminating the tissue damaging side effects.

For the delivery of a DNA vaccine against HBV, we evaluated both a prime and a prime plus boost vaccination protocol. We found that GET parameters of 45V 36 pulses with the addition of moderate heat far exceeded the serum levels of HBsAbs produced by any of the other GET conditions we tested in both the prime and prime plus boost vaccination protocols. These levels were especially elevated in those animals receiving the prime plus boost vaccination schedule and remained stable during the entire 30-week observation period. This suggests that the effects of moderate heat-assisted GET with respect to antibody production

are long lasting. Vaccine longevity is just as critical as compliance, so these results are encouraging. Furthermore, all of the GET conditions we evaluated induced significant HBsAb production compared to injection of pHBsAg alone. Interestingly, though the high-voltage high-pulse condition (45V 72p) showed similar expression levels in our reporter gene experiment, this did not translate to high antibody production in our vaccination study. This could imply that the tissue damage and effects induced by these delivery parameters is too severe to achieve a robust antibody response.

The results of the prime vaccination protocol followed the same trend as the prime plus boost protocol, just at a far lower magnitude. We still observed that GET parameters of 45V 36 pulses with moderate heating induced the highest level of HBsAb production compared to the other tested GET conditions. These results suggest that prime vaccination alone can prompt enduring antibody production in this case, though the prime plus boost protocol was far more robust and more likely to confer long lasting protection. These data were important to collect, as vaccine schedule compliance is a concern among both patients and physicians. An effective vaccine capable of producing sustained protective antibody titers could eliminate the need for multiple booster vaccinations, an appealing solution for all parties. The current vaccination schedule for HBV in infants recommends a series of 3 shots: shortly after birth, aged 1-2 months, and 6-18 months (86,92,93).

Moderate heating increased the overall expression levels achieved by intradermal GET applied at ambient temperature. Moreover, using moderate heat allows for greater penetration depth for the applied electric fields, creating a situation where the uptake of plasmid DNA by professional antigen presenting cells such as dendritic cells is more likely (108-110). This is an

essential aspect of an effective vaccine, mediated by both the cellular and humoral immune systems and dependent upon the specificity for the plasmid-encoded antigen (103,108-111). Where we have historically viewed an intradermal delivery platform as confined to the epidermis and dermis, with the addition of moderate heating it could be possible for aqueous delivery via cutaneous GET to the hypodermis and muscle tissues. This expression pattern was previously demonstrated using reporter genes. The long-lasting HBsAg antibody production we observed after DNA vaccination in our moderately heated GET condition (45V 36p +heat) is evidence that muscle delivery with an intradermal approach is not only possible, but also more than likely occurring. This is favorable, as most vaccines require muscle delivery, though it is unclear if their administration is precise as muscle is an underlying unseen tissue that is not so easily monitored. In addition, intramuscular vaccinations typically administered in the deltoid muscle can cause upper arm injury, bursitis, or other shoulder dysfunctions if not carefully administered (112-115). In infants, intramuscular vaccinations are given in the vastus lateralis located in anterolateral thigh. This site has been deemed the safest intramuscular route, though the middle of the muscle should be the target, not the upper or lower portion, as its adjacency to the muscle branch of the femoral nerve and the lateral circumflex femoral artery (115-117). Intradermal delivery by contrast is visible and therefore easier to ensure correct administration. With moderate heat-assisted GET there are the advantages of muscle delivery with an intradermal approach.

We have previously shown the utility the MEA device for DNA vaccination with a plasmid encoding protective antigen against *B. anthracis* in a murine model as well as for delivering the same plasmid administered in this work against HBV (40,118). Here we expand

upon this original platform and present moderate heat-assisted GET as a novel method with a reconfigured device for intradermal DNA vaccination. Our preliminary results demonstrate that moderate heat-assisted GET yields a significantly higher and longer duration of expression compared to needle-fitted syringe injection alone *in vivo*. As a proof of principle, we show enduring antibody production against HBV *in vivo* over a period of 30 weeks following moderate heat-assisted GET. Compared to our original work, adding moderate heat to GET induced antibody production nearly 8-fold over GET using the MEA at ambient temperature (118). These results indicate the synergy between moderate heat and GET to enhance gene delivery in the skin. Future work should involve characterizing the cellular immune response induced by this approach and a challenge experiment to confirm its protective effects against HBV. Also, these results warrant the consideration of additional vaccinations utilizing plasmid-encoded antigens delivered via moderate heat-assisted GET.

CHAPTER V

MODERATE HEAT-ASSISTED GET FOR PROTEIN REPLACEMENT THERAPY IN THE SKIN

INTRODUCTION

A number of clinical trials have shown positive results for gene-based therapies (119,120). Expression of the delivered gene must be controlled in order to achieve an effective therapeutic outcome while minimizing toxicity and other adverse effects. Though effective, cutaneous GET requires elevated electric fields to penetrate the stratum corneum. A minimally invasive method that does not require the use of high electric fields would facilitate delivery with minimal local discomfort and could potentially reduce or eliminate visible scaring.

To enhance the translatability of GET it is imperative to modify the approach in a manner that could use lower applied voltage while maintaining expression and thereby reducing potential tissue damage. The second issue is to be able to achieve delivery in a minimally invasive manner to the deep- or sub-dermis. One concept to achieve this is to reduce the distance between electrodes. We have previously shown the efficacy a non-invasive MEA for gene electrotransfer to the skin that utilizes a short gap between electrodes (36,80,81,107,118). While this approach improved the outcome with respect to reduced tissue damage, expression was still confined to the epidermis (80). Therefore, alternative strategies were explored to further improve tissue effect as well as to achieve deeper penetration. GET is generally considered a nonthermal process wherein a high voltage pulse is applied creating transient passage through the cell membrane for extracellular deliverables. However, the application of moderate heat can enhance cell membrane fluidity. We therefore, explored the

combination of gene electrotransfer and moderate heating and demonstrated that this could be used to enhance gene delivery to cells and tissues.

While increased temperature can enhance delivery when used together with GET, it is also important to moderate the applied heat so as not to cause tissue damage. It is well established that thermal injury is determined by temperature and duration such that as the temperature is increased, the less time it takes to cause burning or tissue damage (29). We previously established that the optimal intradermal temperature for enhancing GET was 43°C (78). The synergy of this moderate heating protocol and GET resulted in a shorter, lower voltage treatment, presumably more tolerable than a high-voltage high-pulse number approach. Minimizing discomfort is an important consideration for translation of cutaneous deliveries where multiple applications may be necessary such as protein replacement therapy.

Factor IX is a critical clotting protein present in human plasma. Patients suffering from Hemophilia B are variably deficient in the content or activity of this protein, and must receive repeated scheduled injections or intravenous Factor IX to survive. We previously demonstrated the non-invasive delivery of Factor IX using the MEA, where the bulk expression was confined to the skin with a small portion reaching systemic circulation (39). We thus evaluated if using moderate heating as an adjuvant to non-invasive GET may yield higher or more sustained Factor IX protein expression.

The current work demonstrates the utility of moderate heat-assisted GET to the skin using a novel application device based on the design of the MEA. With this device, the addition of an exogenous heating component allows for enhanced uniformity in thermal distribution across the target by way of nine optical fibers connected to an infrared laser. This combination

platform was tested for cutaneous delivery of a therapeutic protein, human Factor IX. The aim of this work was to determine if moderate heat-assisted GET resulted in the expressed protein reaching the blood circulation and for how long this expression was maintained.

For this reason, we suggested the use of moderate heat-assisted GET for delivery of human clotting factor IX. Factor IX is a plasma protein that is critical for thrombosis. Hemophilia B is an X-chromosome linked genetic bleeding disorder in which patients are variably deficient in Factor IX, with ranges below the normal 50-100% plasma protein content (121). It affects approximately one in 30,00 live births and 20% of all Hemophiliacs. Current therapy for Hemophilia B requires frequent dosages administered via injection or central line. These formulations are either recombinant protein or plasma-derived product and have short half-lives. Plasmid DNA by contrast to the aforementioned platforms is self-stable, inexpensive to produce, and innocuous. In the last five years there have been viral gene therapy clinical trials using rAAV to deliver Factor IX (121-123). Though there has been some success in showing extended therapeutic benefit, use of this therapy in patients such as children, those with pre-existing immunity to AAV, and those with hepatitis have been excluded. To mediate this, transient immune suppression or a gain of function Factor IX variants have been suggested to alleviate the unintended side effects from using an AAV vector (68,75,124). For these reasons non-viral alternatives should be considered. Moreover, a minimally invasive route of administration to the skin rather than to the liver, the natural site of Factor IX production, would be ideal in this clinical situation where the risk of severe bleeding is a real concern.

The high expression levels potentiate the use of moderate heat-assisted GET for protein replacement therapy. Sustained protein expression is critical for the treatment of conditions

such as Hemophilia B, where less frequent visits and administrations are not only economical, but also life saving. We demonstrated that moderate heat-assisted GET yielded a significantly higher and longer duration of expression compared to needle-fitted syringe injection alone. In addition, both the pulse number and voltage were reduced by 50% and 23% respectively with the addition of moderate heat, thereby minimizing the perceived pain and superficial damage. Expression was localized in the dermis and muscle layers, thus permitting the ability of therapeutic proteins to reach systemic circulation where they can be most effective.

MATERIALS AND METHODS

Animals

The *in vivo* model for this work was the same as was used in the previous *in vivo* studies presented in Chapters III and IV, as guinea pig skin is similar in both composition and thickness to human skin. Animals used for this study were 8-10-week-old Female Hartley guinea pigs weighing approximately 300-350g at time of procedures. Animal weights were monitored weekly. The animals were housed at the Old Dominion University animal facility and procedures approved by the Old Dominion Institutional Animal Care and Use Committee. IACUC protocol number 17-022 at ODU was followed in accordance for all procedures. All animal subjects were quarantined for a minimum of 7 days following their arrival and prior to the conduction of any experimental procedures.

Plasmids

Endotoxin-free plasmid prepared at 2mg/mL in 0.9% sterile injectable saline of human factor IX expression vector, pNGVL3-CMV-hFIXm1 (generous gift by Dr. Kurachi) was commercially prepared (Aldevron, Fargo, ND). This plasmid operates under a CMV promoter.

Infrared laser heat application

Animals were anesthetized with O_2 containing 2.5-3.0% isoflurane. Prior to treatment both flanks were shaved and washed with mild soap and water to remove loose hair and cleanse the skin of any abundance of oil. This washing procedure was previously described in Chapters III and IV. Moderate heating via a Class IV Laser power supply (LaserMate Group, Inc) via a laser optic fiber (Model: M79L005 Thor Labs Inc.) was applied immediately following a 50µL 2mg/mL intradermal injection of plasmid DNA to the pre-washed and shaven flank skin for a total duration of 30 seconds, after which the laser was switched off. All safety precautions were adhered to while the laser was in operation, including eye protection and barriers.

Electrode design

A novel applicator was constructed that enabled the administration of moderate heating and electric pulses. An infrared radiation emitter was integrated into the applicator. The emitter consists of an optical fiber whose beam is split equally among 9 individual optical fibers utilizing 4 splitter boxes. Main optical fiber is directly connected to a 3-way splitter box, which had three fibers that each wet to another 3-way splitter box. Those nine fibers were then incorporated into the MEA (Figure 3). These fibers are positioned equidistant and centrally between 16 0.5mm gold-plated round tipped pins each spaced 2mm apart, creating a combined 6 by 6 mm moderate heating electrode array (Figure 3). As previously shown, the small size and dielectric material of the optical fiber can be integrated into a GET delivery system. The optical fiber was connected to an infrared semiconductor laser, providing up to 8W of irradiative power at a wavelength of approximately 900 nm. The design of the electrode was modeled after the MEA, delivering 150ms pulses applied in pairs perpendicularly where 4 of 16

electrodes are active at one given time [74]. Following this pulsing pattern, a total of 18 pulses are thereby delivered per round.

Factor IX gene delivery

Animals were anesthetized with O_2 containing 2.5-3.0% isoflurane. As previously described in the other moderate heat-assisted GET studies, animals were pre-shaven and washed with mild soap and water to remove any loose hair or an overabundance of oil. Two 100µg intradermal injections of plasmid DNA encoding human Factor IX were given on separate targets on the same flank. The electrode array was immediately positioned over the injection area with or without exogenous moderate heating. For these studies both 2 and 4 rounds of 18 150ms pulses were delivered, yielding a total of 36 and 72 pulses respectively. In addition, applied voltages of 35V and 45V were assessed. Each site was injected, heated, and pulsed separately and each animal received only one pulsing condition with or without the application of moderate heat. As a positive control group, an intramuscular injection to the hind limb was delivered followed by application of penetrating needle electrodes delivering pulses at 100V/M [119].

Factor IX gene delivery to multiple sites was carried out by prepping the animal subjects in the same way, with skin pre-shaven and washed whilst under a surgical plane of anesthesia. Multiple application sites were given in sets of 2, 3, or 4 100 µg intradermal injections of plasmid DNA encoding human Factor IX. All sites were injected individually, immediately heated then pulsed with 36 pulses at 45V before proceeding to another site. Sites were evenly spaced along the same flank with 1.5 cm distance between each individual site. All sites received the

same heating and pulsing conditions in this experiment where the scalability of Factor IX delivery was evaluated.

Factor IX protein expression analysis

Blood from treated guinea pigs was collected via jugular vein puncture on days 2, 7, 14, 21, 35, 63, and 100. All collections were performed while animals were anesthetized with O_2 containing 2.5-3.0% isoflurane. Animals were monitored until recovery from anesthesia as evidenced by the ability to maintain sternal recumbency. Blood was allowed to clot for at least one hour at room temperature in serum separator tubes. Serum was carefully collected and used to measure clotting factor IX content via a Human Factor IX ELISA kit (ab188393, Abcam).

Statistical analysis

Statistical significance between the groups for the delivery of human Factor IX was determined by 2-way ANOVA with a Tukey-Kramer multiple comparisons test (GraphPad Prism Software, La Jolla, California). Results are expressed as the mean of 5 individuals per group (± SEM). A *P* value less than 0.05 was considered significant.

RESULTS

Factor IX is expressed systemically following moderate heat-assisted GET to the skin

To assess the efficacy of moderate heat-assisted GET for cutaneous delivery of a therapeutic protein, human Factor IX was delivered via this platform in a guinea pig model. Serum was collected from animals following GET. Systemic Factor IX content was reported over a period of 100 days (Figure 16). Moderate heat-assisted GET (45V 36p +heat) yielded the highest protein levels nearing 10ng/mL two weeks after delivery, at least 2- fold over any other treatment condition. Expression waned for this condition and dropped completely by day 35.

The other intradermal conditions, moderately heated or not, followed a similar protein expression pattern, though not at such a high level as the latter. We included an intramuscular GET (IM 100V/cm) condition to serve as a positive control for Factor IX delivery. Conversely to the intradermal GET results, Factor IX protein levels following intramuscular delivery experienced an initial lag and peaked at 8.43ng/mL ± 0.50 at 35 days (*p<0.01*). This increased level of expression was sustained for another two months up to 100 days, ending the observation period.

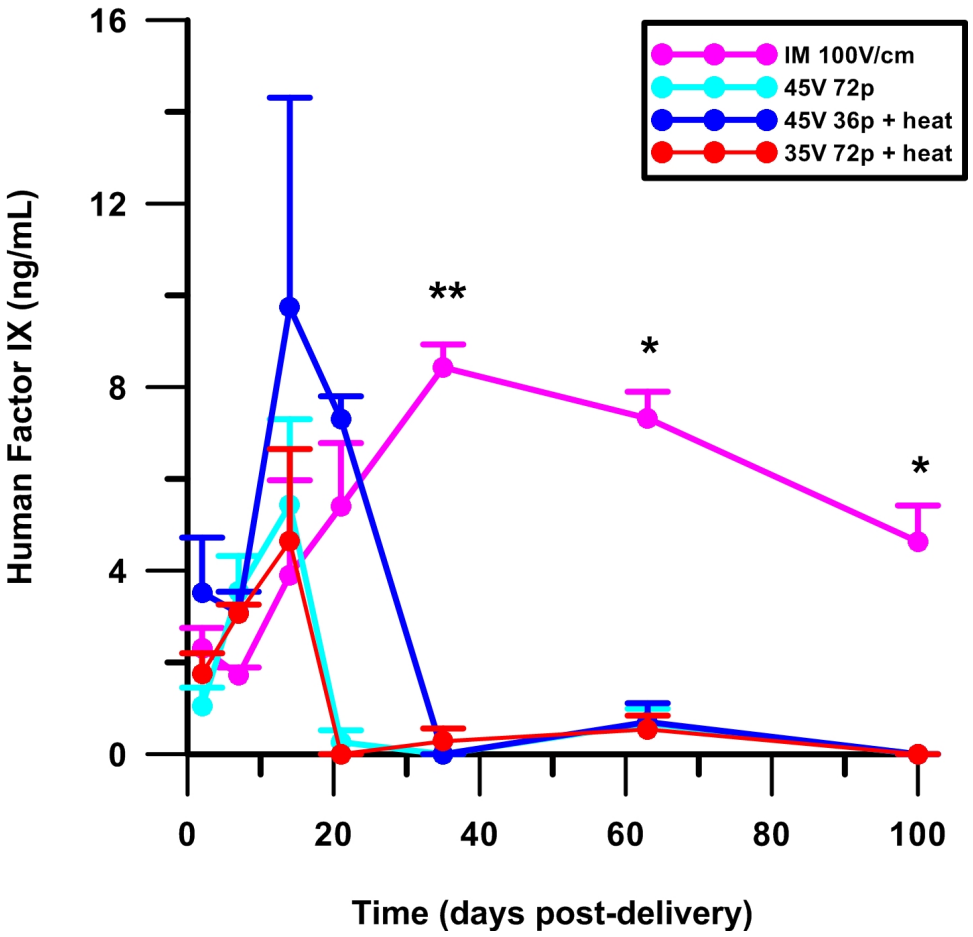

Figure 16. Factor IX expression following moderate heat-assisted and intramuscular GET. Human Factor IX in guinea pig serum. Experimental groups included two intradermal injections of pDNA followed by 72 pulses at 45V (45V 72p), 72 pulses at 35V with moderate heat (35V 72p + heat), 36 pulses at 45V with moderate heating (45V 36p + heat), and a single intramuscular injection of pDNA followed by 8 pulses at 100V/cm. The data represent the average ± SEM, n=5 individuals per group. *,$p<0.05$, **,$p,0.01$.

Multiple site applications enhance Factor IX protein levels

The scalability of cutaneous delivery of human Factor IX via moderate heat-assisted GET was evaluated. Moderate heat-assisted GET was applied in sets of 2, 3, or 4 separate site doses on the same flank in respective animal subjects. Serum was collected from the animals following treatment. Systemic Factor IX protein levels were recorded over a period of 100 days (Figure 17). Utilizing our highest expressing delivery condition from the previous experiment, 45V 36p +heat, we carried out multiple site applications. We observed the highest expression levels nearing 10 ng/mL between 2-3 weeks after delivery across all three experimental groups. Factor IX protein levels then began to wane. The most pronounced drop was in those animals receiving just two application sites, while those receiving 3 or 4 sites maintained elevated Factor IX protein levels for longer. Factor IX protein levels in those animals receiving 3 application sites was significant with respect to those receiving just 2 sites at day 63 ($p<0.05$). Furthermore, in those animals receiving 4 moderate-heat assisted GET application sites, Factor

IX protein levels in the serum were significantly elevated at days 63 and 100 compared to application of 2 sites (*p<0.05, p<0.01*).

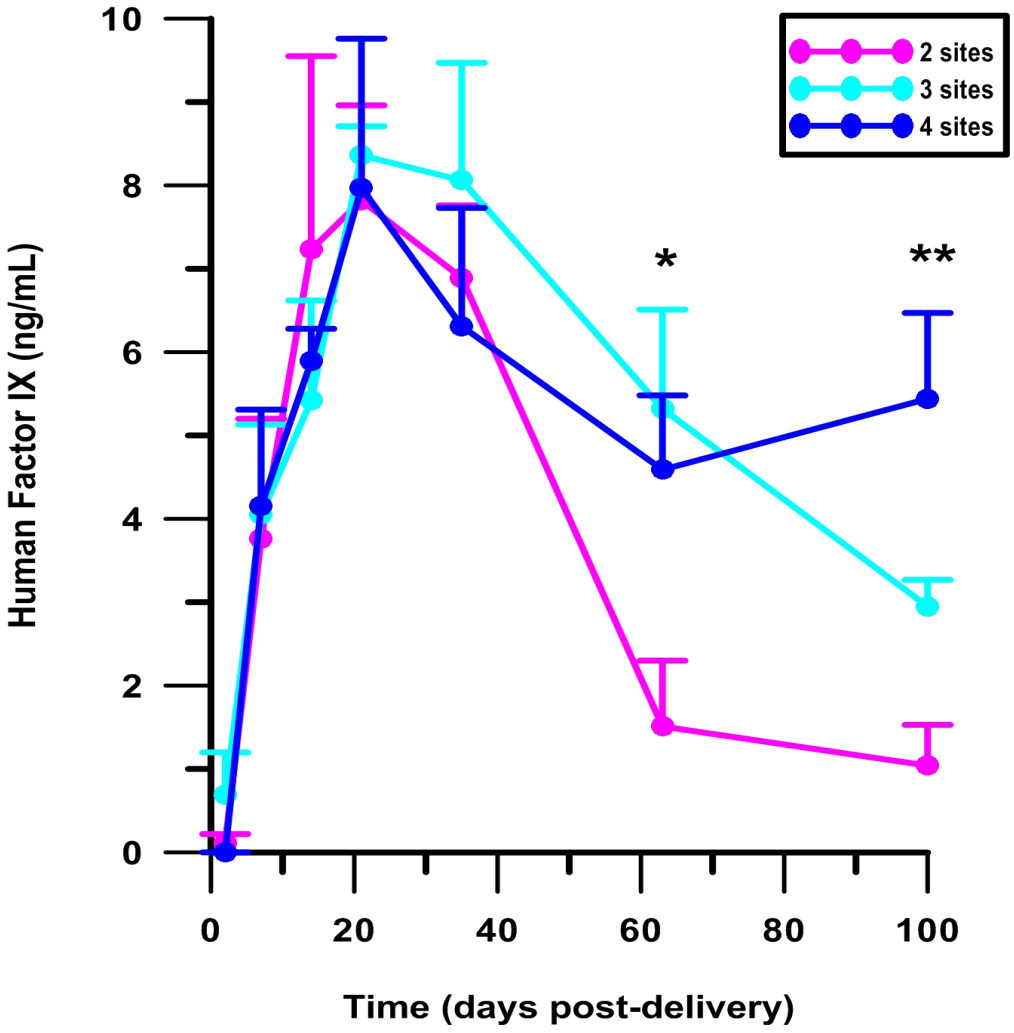

Figure 17. Scalability of Factor IX delivery via moderate heat-assisted GET. Human Factor IX in guinea pig serum. Experimental groups included two, three, or four intradermal injections of pDNA encoding human Factor IX followed by 36 pulses at 45V with moderate heating (45V 36p + heat). The data represent the average ± SEM, n=5 individuals per group. *,*p<0.05*, **,*p,0.01*.

DISCUSSION

The results from Chapter III highlight the high expression levels achieved with the use of moderate heat-assisted GET for the delivery of reporter genes. Here we suggest this platform for protein replacement therapy. Elevated and long-lived protein expression is critical for the treatment of conditions such as Hemophilia B, where less frequent visits mean fewer injections or infusions carrying a lower risk of severe bleeding. In Chapter III, we demonstrated that moderate heat-assisted GET yielded a significantly higher and longer duration of expression compared to needle-fitted syringe injection alone, where both the pulse number and voltage were reduced by 50% and 23% respectively with the addition of moderate heating. Expression distribution was localized in the dermis and muscle layers, which we surmise would be advantageous for applications such as Factor IX protein replacement therapy, for achieving sustained protein expression levels.

We included an intramuscular GET delivery dose in our Factor IX experiment as a positive control since this platform has been shown to yield long-term sustained transgene expression (125-127). We observed just that in our study, with Factor IX protein levels peaking 35 days after IM GET delivery and waning gradually out to 100 days of continued observation. At day 35, expression among all intradermal delivery groups dropped. In comparing this pattern to our intradermal GET delivery, peak expression was observed 14 days following moderate heat-assisted GET at 45V 72 pulses, where expression levels after IM GET were 2.5-fold lower at this same time point ($p < 0.05$).

While these results are very promising, we do recognize that actual protein levels are still low. Normal physiological level of Factor IX is 100ng/mL, so adjustments would need to be

made to increase the amount of protein that actually reaches circulation, otherwise this could only be useful for patients suffering from mild Hemophilia B, where Factor IX levels are greater than 5% but less than 50% total plasma protein content. We addressed this concern by evaluating the scalability of moderate heat-assisted GET for Factor IX delivery. In this case, we selected our highest expressing GET condition (45V 36p +heat) and tested the effects of additional sites on Factor IX protein levels in the serum. We observed peak protein levels in all experimental groups of 2, 3, and 4 injection sites within 21 days after initial delivery. Like our initial experiment, protein expression dropped sharply after peaking in those animals receiving just two application sites. Interestingly, protein levels remained significantly elevated in animals receiving 3 or 4 moderate heat-assisted GET application sites with prolonged Factor IX expression up to 100 days after delivery. This is encouraging and suggests the potential to scale up to enhance the amount of protein that reaches systemic circulation. Beyond simply adding more injection sites, modifications to increase the delivery surface area could be achieved by designing a larger applicator. Moreover, alternative therapeutic purposes where smaller amounts or weight of product are needed, such as growth hormone delivery, could also be an appropriate application for this technology (128,129).

CHAPTER VI

CONCLUSIONS

In this study we evaluated the use of a novel device performing moderate heat-assisted GET for intradermal delivery in two applications: DNA vaccination and protein replacement therapy. Optimization of the infrared laser as the exogenous moderate heating source was carried out in an *ex vivo* pig skin model as well as a 3D human skin model before *in vivo* studies were performed. The guinea pig was used as the animal model for these studies. *In vivo* moderate heating parameters were defined as the application of heat for 30 seconds to reach an intradermal temperature of 43°C, which was maintained for approximately 20 seconds after the removal of the exogenous heating source.

Delivery of reporter genes via moderate heat-assisted GET was tested before moving to therapeutic agents to assess ideal parameters for achieving high expression levels. We found that a high-voltage low-pulse number condition plus moderate heating (45V 36p +heat) resulted in a significant increase in luciferase expression compared to any of the other tested conditions. These results were similar to a positive control condition, high-voltage high-pulse number (45V 72p), which displayed burning at the treatment site and therefore deemed unsuitable for translation to non-life-threatening applications. Conversely, despite displaying high expression levels, no skin damage was observed in the moderately heated condition. Expression distribution was found to extend to the deep dermis and muscle of those animals receiving high-voltage low-pulse number GET with the addition of moderate heat. This suggested that an intradermal approach could result in muscle gene delivery and precipitated efforts to demonstrate its efficacy in therapeutic applications.

We evaluated the utility of moderate heat-assisted GET for the delivery of a DNA vaccine against HBV. We previously found that a plasmid dose of 100µL at 1mg/mL induced the highest reporter gene expression, so this dose was utilized for the DNA vaccination study. A plasmid encoding Hepatitis B surface antigen was given in a prime-boost and prime only vaccination protocol. Our data indicate that neutralizing antibodies to HBsAg were elevated in serum 230-fold over injection of plasmid DNA alone in those animals receiving our highest expressing condition (45V 36p +heat). Antibody titers peaked 18 weeks after a prime-boost vaccination protocol and remained elevated over all other tested conditions for the duration of the 30-week observation period. We did not observe this high level of HBsAb in our high-voltage high-pulse positive control condition (45V 72p), suggesting the damage observed with this condition was too severe to generate a robust antibody response. Due to the high generation of antibodies following the prime-boost protocol, we evaluated antibody generation with this platform using a prime only protocol. Vaccine compliance is a concern, so the advantage of a single dose vaccination compared to a boost protocol is less vaccinations, which may be an easier recommendation to follow. In our prime only protocol, we found that antibody titers were generated in all of our GET conditions, with the moderately heated high-voltage low-pulse number condition (45V 36p +heat) being the highest for the duration of the 30-week observation period. Antibody titers were still much lower in this case compared to the prime-boost protocol, suggesting the later to be more effective at conferring immunity.

Finally, we evaluated the efficacy of moderate heat-assisted GET for the delivery of human Factor IX to the skin. Like our previous *in vivo* studies, a guinea pig model was used for this work. Here we show that systemic Factor IX protein generation was induced following our

moderately heated high-voltage low-pulse number GET condition (45V 36P +heat). These levels peaked at 14 days near 10ng/mL, ten-fold below therapeutic levels. We included an intramuscular GET delivery condition as a positive control. Factor IX expression peaked at 35 days following GET as the intradermal GET conditions were waning. Taken together this is encouraging, suggesting the utility of a two-armed delivery approach using both IM GET and moderate heat-assisted GET, to achieve both the early Factor IX expression with moderate heat-assisted GET and the lagged but sustained levels achieved with IM GET. While these results are very promising, we do recognize that actual protein levels are still low. Normal physiological level of Factor IX is 100ng/mL, therefore adjustments would need to be made to increase the amount of protein that actually reaches circulation. To accomplish this, we scaled up our delivery approach by evaluating overall Factor IX delivery using multiple delivery sites. These data suggest the platform is scalable, and therefore updates to the electrode configuration could further enhance the utility of this technology. An infrared laser was chosen as the heating source in this case due to its speed and precision at which the target temperature could be reached and specifically applied. However, in the future, utilizing different albeit slower exogenous heating sources such as LED, induction, or convection could be advantageous towards creating a more user-friendly platform in general.

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Selected Publications

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